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PROTEIN BIOSYNTHESIS IN THE THYROID GLAND

A Study of Control Mechanisms

by

Dorothy J. Begg, B.Sc.

Preliminary investigations were carried out to devise means of estimating the amounts of RNA and DNA in the thyroid gland. A study was then made of the nucleic acid, protein and phospholipid content of the thyroid glands of different mammalian species, and relationships to thyroid weight and total body weight established. It was found that, with increasing size of mammal, the concentration of protein in the thyroid gland increases, whereas there is a diminution in the concentration of RNA and, to a smaller extent, of DNA and phospholipid. These findings can be correlated with an increase in follicle size in larger mammals, which results in more colloid in proportion to the number of cells. Calculations made from the data show that the amount of protein in the thyroid glands of various mammals is maintained at a constant proportion of body weight. However, the number of cells in the thyroid gland does not increase in parallel with the size of the species and the larger mammals also have less RNA per cell. These observations suggest that the turnover of protein in the thyroid glands of larger mammals may be less rapid than in smaller mammals.

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Isotopic studies were then carried out using tissue slices and purified nuclei prepared from sheep thyroid glands to study the effects of TSH on thyroid RNA and protein metabolism. When slices of sheep-thyroid tissue were incubated with TSH and (^{14}C) adenine, it was found that TSH caused an increase in labelling of nuclear RNA and subsequently of cytoplasmic RNA, and that this increase was detectable in all thyroid RNA fractions after 3 hr. incubation. The incorporation of (^{14}C) adenine into the RNA of isolated sheep-thyroid nuclei in vitro was also found to be stimulated by the presence of TSH in the incubation medium. Rat-liver nuclei were not responsive to TSH under the same incubation conditions, and addition of protein (bovine serum albumin) to the incubation medium had no effect on adenine uptake into thyroid nuclear RNA. TSH also stimulated the uptake of (α - ^{32}P) UTP into RNA in the presence of the other unlabelled ribonucleoside triphosphates.

The effect of TSH on adenine uptake into thyroid RNA could be inhibited by treatment of the slices or the isolated nuclei with puromycin or actinomycin D. These findings suggest that the earliest effects of TSH on thyroid RNA metabolism involve the synthesis of both nuclear RNA and protein. However, addition of TSH to an incubation medium

containing isolated sheep-thyroid nuclei had no significant effect on (^{14}C) leucine uptake into nuclear protein under conditions where it stimulated (^{14}C) adenine uptake into nuclear RNA. This suggests that the protein apparently involved in stimulation of nuclear RNA synthesis by TSH must contribute to only a small proportion of the total nuclear protein.

Purified sheep-thyroid nuclei were then fractionated either by the procedure of Weiss (1960) or by that of Ramuz, Doly, Mandel and Chambon (1965) and assays of DNA-dependent RNA polymerase activity carried out using the three nuclear enzyme fractions obtained. Some properties of these polymerase enzyme fractions were then characterized and the effect of TSH on DNA-dependent RNA polymerase activity examined. Direct addition of TSH at the start of incubation to assay mixtures containing these enzyme fractions had no effect on DNA-dependent RNA polymerase activity. Treatment of isolated sheep-thyroid nuclei with TSH under conditions where it is known to stimulate the uptake of (^{14}C) adenine into nuclear RNA was found to increase the ability of the "Ramuz" aggregate enzyme preparation to incorporate labelled UMP from UTP but not labelled AMP from ATP into RNA. However, no effect of TSH was apparent when Mg^{2+} replaced Mn^{2+} during polymerase assay. Puromycin treatment of sheep-thyroid

nuclei, which had been incubated in the presence or absence of TSH prior to enzyme preparation, was found to decrease the DNA-dependent RNA polymerase activities of the enzyme fractions prepared by the procedure of Ramuz et al. (1965).

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ABBREVIATIONS

TSH	(thyroid-stimulating hormone) Thyrotropin
DNA	Deoxyribonucleic acid
RNA	Ribonucleic acid
tRNA	Transfer ribonucleic acid
sRNA	Soluble ribonucleic acid
DNase	Deoxyribonuclease
RNase	Ribonuclease
ATP	Adenosine-5'-triphosphate
CTP	Cytidine-5'-triphosphate
GTP	Guanosine-5'-triphosphate
UTP	Uridine-5'-triphosphate
AMP	Adenosine-5'-monophosphate
UMP	Uridine-5'-monophosphate
(α - ^{32}P)UTP	Uridine-5'-triphosphate with ^{32}P in the phosphate residue adjacent to the uridine
($\beta\gamma$ - ^{32}P)UTP	Uridine-5'-triphosphate with ^{32}P in the phosphate residues distant from the uridine
(α - ^{32}P)UMP	Uridine-5'-monophosphate with ^{32}P in the phosphate residue adjacent to the uridine
poly A	Polyribonucleotide strands containing only adenylate residues
poly U	Polyribonucleotide strands containing only uridylate residues
mRNA	Messenger ribonucleic acid

NAD	Nicotinamide-adenine dinucleotide
NADP	Nicotinamide-adenine dinucleotide phosphate
P_i, PP_i	Orthophosphate, pyrophosphate
tris	2-Amino-2-hydroxymethylpropane-1,3-diol
EDTA	Ethylenediaminetetra-acetate

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GENERAL INTRODUCTION

GENERAL INTRODUCTION

All cellular functions are ultimately dependent on gene activity. In each individual there is considerable diversity of function, structure and chemical composition among the cells of different tissues and to a lesser degree within a particular tissue itself. All these cells however basically have identical sets of genes i.e. deoxyribonucleic acid (DNA). It follows therefore that there must exist some mechanism which controls the phenotypic expression of genes in the developmental process. This might be explained by the assumption either of uniform gene activity in all cells and a cell-specific selection of genetic messages at the cytoplasmic level or differential gene activity patterns.

Little is yet known of the control mechanisms involved in cytodifferentiation. However it would appear that cellular responses to particular stimuli, such as the presence of hormonal molecules, result from genetic programming of specific intracellular reactions to these stimuli, and thus that hormonal control of metabolism is superimposed on basic regulatory mechanisms. It has been known for some time that hormones can affect, either directly or indirectly, the nucleic acid

metabolism of their target organs (Leslie, 1955), and that they can also affect the levels of cellular enzymes and protein in these particular tissues (Knox, Auerbach and Lin, 1956).

With the recent advances in the elucidation of the steps involved in cellular protein synthesis, and particularly the nature of the involvement of nucleic acids in this process (see Korner, 1964; Moldave, 1965 for reviews), it has become possible to study hormonal effects at subcellular levels in an attempt to explain, on a molecular basis, the ability of hormones to influence both the synthesis and activity of other biological molecules.

For the present investigations into this problem studies have been carried out on the mode of action of the hormone thyrotropin (TSH) on the nucleic acid and protein metabolism of its target organ, the thyroid gland. The main function of the thyroid gland appears to be that of elaborating, storing and secreting its own peculiar iodine-bearing hormones, the elaboration and release of which is stimulated by TSH. Before one can discuss the known effects of thyrotropin on the thyroid, a detailed account of thyroid cell metabolism is essential for an understanding of these actions since most thyroid metabolic processes undergo some change upon hormone administration.

Morphology and Cytology of the Thyroid Gland

The thyroid gland consists of a large number of follicles or acini (Fig. 1). These have lost all luminal connection with other parts of the body and may be considered from both structural and functional points of view as the primary or secretory units of the organ. The cells of the follicles are the sites of hormone synthesis; the follicles are the storage depots. In the normal human adult gland the follicles are roughly spherical and vary considerably in size, the average diameter being about 300 μ . The wall consists of a continuous epithelium, one cell deep, the parenchyma of the thyroid. In the resting gland, the cells are flattened, but on stimulation with TSH they increase in height and become columnar. Within the follicle and filling its lumen is the homogeneous colloid which consists mainly of protein material, the principal protein being thyroglobulin.

The fine structure of the thyroid follicular cell is shown diagrammatically in Fig. 2. Thyroid follicular cells viewed in the electron microscope are basically similar to the epithelial cells of exocrine glands engaged in protein

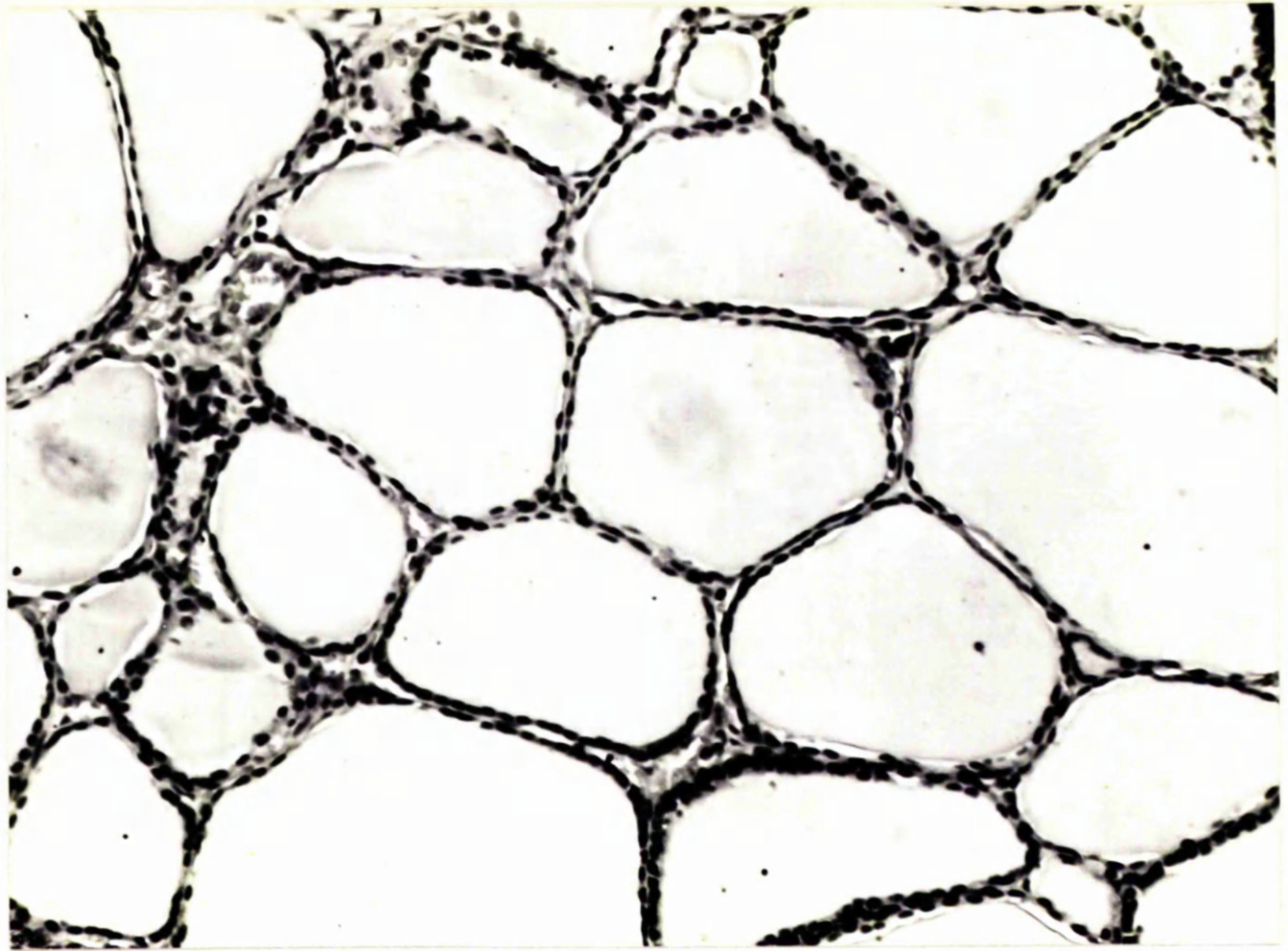


Fig. 1

Human thyroid gland (x275) H. and E. The acini are filled with uniformly staining colloid and are lined by a rather flattened cuboidal epithelium.
(Reproduced by kind permission of Dr. W.P. Duguid of the Pathology Department, Royal Infirmary, Glasgow.)

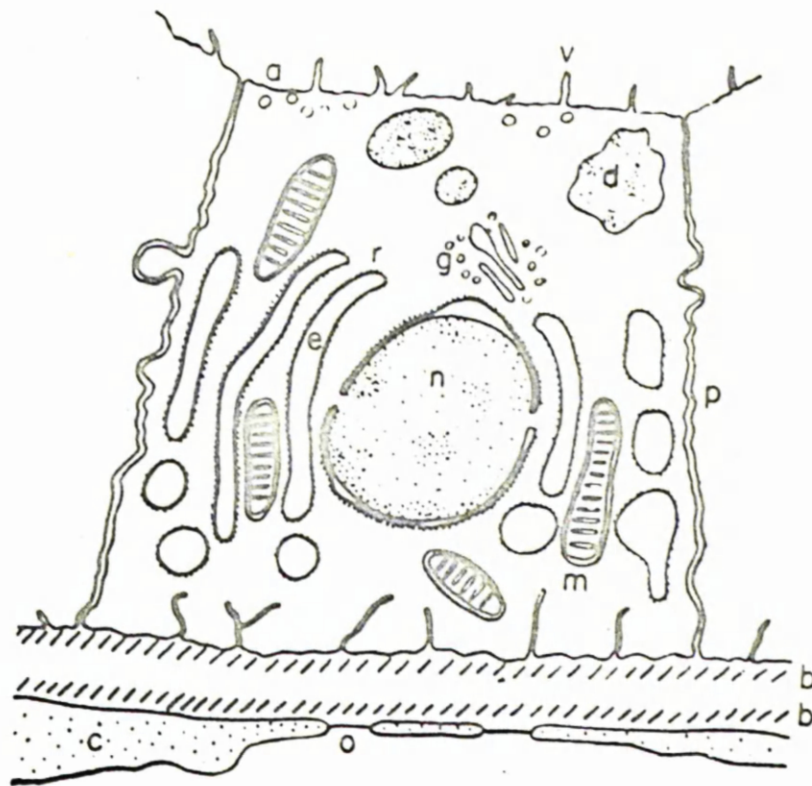


Fig. 2

Schematic Drawing of a Thyroid Follicular Cell

(From Ekholm and Sjöstrand, 1957)

- | | |
|----------------------------|--|
| a - apical surface of cell | r - ribosomes |
| e - endoplasmic reticulum | g - Golgi apparatus |
| d - "secretion droplets" | m - mitochondrion |
| v - microvillus | p - plasma membrane |
| c - capillary cell | b - basement membrane |
| n - nucleus | o - open "pore" in
endothelial cell |

secretion. Microvilli occur along their apical surface, they have a well-developed Golgi apparatus associated with secretory droplets located between the nucleus and the apical surface, they have an extensive ergastoplasmic network deployed throughout the cell, and they synthesize protein and secrete it at the apical surface into vesicles. Unlike ordinary exocrine cells, however, they have an additional secretory function since they act as endocrine cells by releasing thyroid hormone into the perifollicular capillaries. Thyroid hormones are stored in the colloid as part of thyroglobulin and hydrolysis must occur before these hormones, thyroxine and 3-5-3'triiodothyronine, can escape into the circulation. The thyroid cells are separated from the capillaries by two layers of basement membrane but minute pores in the endothelial lining of the capillaries allow plasma to come into direct contact with the basement membrane permitting diffusion of materials in and out of the acinar cells.

Biosynthesis and Release of Thyroid Hormones

This has been recently reviewed by De Groot (1965) and only a brief account of the topic will be given here. In

outline, the basic aspects of the process are shown in Fig. 3. The thyroid gland accumulates inorganic iodide and establishes a concentration gradient between gland and plasma. The accumulated or 'trapped' iodide is oxidized and bound to tyrosine present in peptide linkage in thyroglobulin. The iodinated tyrosines, mono- and di-iodotyrosine, couple to form the thyronine molecules, thyroxine and 3-5-3'triiodo-thyronine, which are still held within the peptide chain of thyroglobulin. These iodotyrosines and iodothyronines are subsequently liberated by thyroid proteolytic enzymes. The iodothyronines are released into the plasma; the iodotyrosines are deiodinated, and their iodide is reutilized within the gland. In the peripheral tissues the iodothyronines exert their metabolic effect, and in turn are degraded. Their iodide is released to mix in the plasma pool with the newly ingested iodide and to traverse this cycle once again.

(1) Iodide-concentrating process.

This process can be arbitrarily divided into two stages:-
iodide transport and iodide binding.

Iodide Transport - This has recently been reviewed by Wolff (1964_a). The thyroidal transport process results in the concentration of free iodide inside the lumen of the follicle (Pitt-Rivers and Trotter, 1953). Although this localization might initially suggest that active transport occurs at the apical margin of the cell transferring iodide into the colloid space, data are available which locate the concentration process at the cell base. The most direct evidence for this is the concentration of free iodide by isolated thyroid cells (Tong, Kerkof and Chaikoff, 1962). The exact mechanism of transport remains unknown. However, it has been shown that the process is energy-dependent (Freinkel and Ingbar, 1955), and can be saturated by iodide (Wyngaarden, Stanbury and Rapp, 1953). Wolff and Maurey (1958) observed that the process is K^+ -dependent, and a relationship to the ATPase activity of cell membranes (Turkington, 1962) and of thyroid homogenates (Wolff and Halmi, 1963) has been shown. It has been proposed that ATPase activity is related to an energy-requiring step in iodide transport. Vilkki (1962) found that iodide forms a complex with thyroidal lecithin and the kinetics of iodide-

binding to thyroidal phospholipids have been studied by Schneider and Wolff (1965).

Site of Iodide Binding - The locus of binding of iodide to tyrosine is not yet clear; however it is generally accepted that iodination occurs on tyrosine bound in peptide linkage rather than on free tyrosine that is subsequently incorporated into protein. The most conclusive evidence for this sequence is the absence of specific iodotyrosine-activating enzymes or receptor tRNA in the thyroid gland (Alexander, 1964; Cartouzou, Aquaron and Lissitzky, 1964), and the inhibition of protein synthesis by puromycin without cessation of protein iodination (Soodak, Maloof and Sato, 1964; Seed and Goldberg, 1965). Radioautography using ^{131}I clearly demonstrates the predominant localization of protein-bound ^{131}I within the follicular lumen even a few seconds after administration of tracer isotope (Wollman and Wodinsky, 1955; Pitt-Rivers, Niven and Young, 1964). It is apparent however, in many radioautographs, that protein-bound ^{131}I first appears in rings round the periphery of the luminal space later diffusing to give a uniform blackening of the colloid, and it seems that iodination does occur initially near the colloid-cell interface

in proximity to the microvilli (Stein and Gross, 1964; Williams and Vickery, 1965).

(2) Mechanism of Iodotyrosine Formation

Iodination in mitochondrial and microsomal preparations derived from sheep and calf-thyroid tissue has been investigated by De Groot and his co-workers (De Groot and Carvalho, 1960; De Groot and Davis, 1961; De Groot, Thomson and Dunn, 1965). Both animal tissues contain an enzyme capable of binding ^{131}I to tyrosine in particulate protein or to soluble tyrosine (De Groot and Davis, 1962; De Groot et al., 1965). The reactions are augmented by H_2O_2 and are inhibited by SH-compounds especially antithyroid drugs. The evidence available suggests that formation of H_2O_2 or some other biological oxidizer is used to oxidize iodide to a more reactive state for iodination of tyrosine. However there is no proof as yet for a distinct 'tyrosine-iodinase' enzyme for the production of mono- and di-iodotyrosine.

(3) Sequence of Formation of Iodotyrosines and Iodothyronines

From in vivo studies it has been shown that mono-iodotyrosine is the precursor of diiodotyrosine (Taurog, Tong and Chaikoff, 1958; Pitt-Rivers, 1962). Thyroxine and 3-5-3'tri-

iodothyronine are then formed by the coupling of two iodotyrosine residues with the splitting out of the residue of one alanine side-chain (Johnson and Tewkesbury, 1942; Plaskett and Barnaby, 1964). Thyroglobulin serves as the matrix within which the coupling occurs in the formation of iodothyronines. It is in addition the storage site of thyroid hormone.

(4) Biosynthesis of Thyroglobulin

Thyroglobulin has been characterized in several mammals as a glycoprotein of molecular weight approximately 650,000. From in vitro studies using sheep-thyroid slices, Seed, Goldberg and co-workers have demonstrated, in a series of papers, the mechanism of formation of the polypeptide chains of thyroglobulin. When radioactive amino acids are incorporated into thyroglobulin (19S in molecular size) by thyroid slices label is detected in more slowly sedimenting proteins (3 - 8S and 12S) before it appears in thyroglobulin (Seed and Goldberg, 1963, 1965; Lissitzky, Roques, Torresani, Simon and Bouchilloux, 1964). Edelhoch and Lippoldt (1960, 1962) have shown that thyroglobulin is composed of two half-molecules (12S) which in turn consist of two polypeptide chains (3 - 6S) linked by

disulphide bonds. This suggests that the more slowly sedimenting radioactive proteins are sub-units of thyroglobulin, and possibly represent intermediate stages in thyroglobulin biosynthesis. This postulate is supported by results of kinetic experiments and by the physical and immunological properties of these proteins (Seed and Goldberg, 1963, 1965; Lissitzky et al, 1964; Sellin and Goldberg, 1965).

There is also some evidence that thyroglobulin is unstable when first formed. Thyroglobulin into which radioactive amino acids and iodine have been incorporated in vitro sediments more slowly and is more labile to dissociation into 12S half-molecules than pre-existing thyroglobulin. Since iodination occurs after formation of the polypeptide backbone (Taurog and Howells, 1964; Seed and Goldberg, 1965; Tishler and Ingbar, 1965) and since synthesis of its carbohydrate and polypeptide components are separate stages in thyroglobulin biosynthesis (Spiro and Spiro, 1965a), the greater ease of breakdown of the newly formed thyroglobulin, poor in iodine and perhaps in carbohydrate, could be ascribed to its less compact tertiary structure i.e. a manifestation of its relative biochemical

immaturity. The chemical alterations which confer 'maturity' upon thyroglobulin may be several, including iodination, carbohydrate addition and possibly interchain covalent bond formation. In this connection it should be noted that a 27S iodoprotein, which appears to be a polymer of thyroglobulin sub-units, has been isolated from human and bovine thyroid glands (Salvatore, Vecchio, Salvatore, Cahnmann and Robbins, 1965). This iodoprotein appears to have a higher iodine content than thyroglobulin, and may bear a similar relationship to thyroglobulin as does haemosiderin to ferritin - iron-storage proteins.

(5) Release of Thyroid Hormone from the Thyroid

Once formed by coupling, iodothyronines are either stored in thyroglobulin or released into the bloodstream. Release is accomplished by a series of proteases and peptidases which as yet have not been well characterized (Litonjua, 1960; McQuillan, Mathews and Trikojus, 1961; Poffenbarger, Powell and Deiss, 1963). Little is known of the intracellular location of this process. However, the presence in many cells of organelles called lysosomes (Applemans, Wattiaux and De Duve,

1955) which contain a variety of proteolytic enzymes, leads to the suggestion that these organelles, if they occur in the thyroid, may be involved in the proteolysis of thyroglobulin. Droplets which stain like colloid have been detected in the cytoplasm of thyroid follicular epithelia after TSH stimulation (Wollman, Spicer and Burstone, 1964; Wetzel, Spicer and Wollman, 1965). Acid phosphatase and esterase were localized in these droplets and, in addition, in granules largely located around the nucleus. These hydrolytic enzymes are characteristic of lysosomes (De Duve, 1959). Thus it appears that thyroid cells contain preformed hydrolytic enzymes associated with lysosomes. When the gland is stimulated by TSH intracellular droplets are formed from colloid derived from the vesicles and hydrolytic enzymes are transferred from the lysosomes to these droplets. These droplets may be the intracellular organelles for the hydrolysis of colloid and liberation of thyroid hormone prior to release into the bloodstream.

Further evidence for this process comes from studies of Balasubramaniam, Deiss, Tan and Rowell (1965). Dog thyroid glands were prelabelled with ^{131}I and the animals

killed shortly after TSH treatment. Differential centrifugation of the homogenized thyroids showed the highest labelling of protein-bound ^{131}I in the fraction where lysosomes would sediment if present in the thyroid homogenate.

Role of Thyrotropin

The first demonstration that the activity of the thyroid gland was dependent on a hormone secreted by the pituitary gland was made by Smith and Smith (1922), when they showed that, in hypophysectomized tadpoles, the atrophic thyroid gland could be made to hypertrophy by injections of bovine anterior pituitary tissue. Smith (1926) subsequently developed a technique for hypophysectomizing the rat and was able to demonstrate the stimulatory action of pituitary extracts on the thyroid of the hypophysectomized animal. It was subsequently shown that functioning of the normal thyroid gland at a physiologically significant level is dependent upon the stimulatory effect of the hormone thyrotropin from the anterior pituitary. Thyrotropin is secreted by a specific type of basophil cell in the anterior pituitary (Purves and Griesbach, 1951) and is a glycoprotein of molecular weight about 25,000, but its structure

and amino acid sequence have not yet been characterized.

Control of TSH Secretion

It was shown by Aron, Caulaert and Stahl (1931) that lack of thyroid hormone stimulated the secretion of TSH from the pituitary, while an excess of hormone was inhibitory, and Von Euler and Holmgren (1956) found that thyroxine injected into the pituitary gland caused decreased secretion of thyrotropin from the gland. Bogdanove and Crabill (1961) implanted thyroid tissue directly into the pituitary and found that it induced a local response in the thyrotropin-producing cells. Thus the control mechanism of action of thyroid hormone in inhibiting thyrotropin secretion is seen to be one of negative feedback in the sense that thyroid hormone acts against its own production.

The rate of thyroid secretion also resembles that of gonadal and adrenal secretion in being responsive to stimuli from the external environment which act primarily on the central nervous system. However these responses of thyroid secretion rate to environmental stimuli do not always appear to be produced in the same way. Some are apparently produced by alterations in the rate of degradation and excretion of thyroid hormone, and in

others there appears to be a 'hypothalamic effect' on the TSH-producing cells in the anterior pituitary resulting in a change in the level at which the thyroid hormone is regulated. In connection with the latter, Guillemin and co-workers (Guillemin, Yamazaki, Jutisz and Sakiz, 1962; Guillemin, Sakiz and Ward, 1965) have demonstrated the existence, in crude extracts of hypothalamic tissues, of a polypeptide substance, thyrotropin-releasing factor (TRF) which specifically stimulates the secretion of TSH from the anterior pituitary in vivo.

Thyrotropin does not appear to play any essential part in the processes by which the thyroid gland elaborates and secretes its hormone; instead it accelerates the rates of processes of which the thyroid cells are intrinsically capable. Reports have been made of stimulation by TSH of many metabolic processes, and at the moment no particular function can be said to be the primary site of action of TSH; it would appear that thyrotropin acts on the thyroid cell to modify all its aspects and functions. A brief summary is presented of the effects of TSH on the structure and on various metabolic

pathways of the thyroid gland.

(1) Morphological Changes produced by TSH

Following the exhibition of TSH, there is an increase in the vascularity of the thyroid gland and a prompt augmentation of follicular cell height. Concomitantly, nuclear volume increases and later on mitotic figures become more frequent. Sustained hyperplasia and hypertrophy result in an increase in glandular weight.

Of possible greater relevance to the more fundamental action of thyrotropin are the biochemical changes underlying these gross morphological effects. Biochemical studies have been carried out extensively using both in vitro and in vivo systems. On the basis of in vitro studies, TSH effects may generally be divided into two categories: (a) those that are elicited by TSH in systems unsupplemented with nutrients; these reflect endogenous events uncoupled to exogenous contributions to net energy balance, and (b) those reflecting changes in assimilative function due to TSH; these can be demonstrated only in supplemented systems. Both types are mentioned below.

(2) Metabolic Changes produced by TSH

(a) Elevation of Oxygen Consumption This could be observed at early times after the addition of TSH to sheep-thyroid slices incubated in the absence of any exogenous metabolites (Freinkel, 1957).

(b) Effects on Salt and Water Metabolism Two hours after a subcutaneous injection of TSH into guinea pigs, there is an increase in the water and potassium content of the thyroid (Gedda, 1960). Solomon (1961) showed an increase in ^{24}Na uptake in the chick very rapidly after thyrotropin treatment. This may reflect the net transfer of sodium from the lumen into the cell on thyroglobulin proteolysis, or an increase in permeability of the cell membrane affecting ion transport.

(c) Effects on Carbohydrate Metabolism TSH added in vitro promotes the assimilation of glucose from the suspending medium by sheep-thyroid slices (Field, Pastan, Johnson and Herring, 1960; Freinkel, 1960). Attempts to show separate effects of TSH upon alternative pathways of glucose metabolism have been limited to analyses of different rates of evolution of $^{14}\text{CO}_2$ from (1- ^{14}C) glucose and (6- ^{14}C) glucose (Dumont, 1961;

Field et al, 1960; Schussler and Ingbar, 1961). Because of the striking increase in $^{14}\text{CO}_2$ evolution from (1- ^{14}C) glucose which can be demonstrated in vitro in as little as five minutes (Field et al, 1960; Dumont, 1961), it has been postulated that thyrotropin selectively activates the hexose monophosphate pathway, and that this may constitute the primary locus of TSH action (Field et al, 1960). There is an objection to this proposal in view of the well-documented reactions to TSH which occur in unsupplemented systems despite the very low levels of intrathyroidal glycogen.

It has been suggested that the increase in glucose oxidation is secondary to changes in pyridine nucleotide concentration since a conversion of NAD to NADP would promote glucose oxidation by the hexose monophosphate pathway. Studies have indicated that TSH does not alter the oxidation of NADPH by thyroid mitochondrial-microsomal preparations although the hormone does increase levels of NADP in thyroid slices during incubation in a medium containing glucose (Field, Pastan, Herring and Johnson, 1961) and also in the absence of glucose (Pastan, Herring

and Field, 1961). Thyrotropin causes a rise in NADP, an equivalent fall in NAD, and negligible changes in NADPH and NADH. On the basis of the reciprocal alterations in the concentrations of NADP and NAD, Pastan et al., (1961) have suggested that the increased NADP is derived from a conversion of NAD possibly through NAD kinase and that the changes due to TSH follow from enhanced availability of NADP. However, this cannot be due to de novo synthesis of NAD kinase since, in the presence of puromycin at a concentration sufficient to inhibit the uptake of (14 C) leucine into thyroid protein, the TSH effect on glucose oxidation is still demonstrable (Field, Johnson, Kendig and Pastan, 1963). Also studies of Field, Remer and Epstein (1965) suggest that NAD is not rate-limiting in the formation of NADP by the enzyme NAD kinase because the effect of TSH on thyroid glucose oxidation is only significantly reduced when there are very marked reductions of NAD. Subsequent investigations carried out on this effect of TSH have not yet clarified the situation. It has also been shown that TSH augments the oxidation of other metabolites such as acetate, pyruvate, gluconate and gluconolactone in vitro.

(d) Effects on Phosphorus and Phospholipid Metabolism

Freinkel (1963) has shown that shortly after the addition of TSH to tissue slices intrathyroidal inorganic phosphate was invariably augmented. This could not be attributed solely to an influx of phosphate from the external medium, and in consequence thyroidal phosphatase activity must occur very soon after TSH administration. The nature of the phosphatases involved have not yet been elucidated. Turkington (1962) has demonstrated an increase in membrane phosphatase activity shortly after TSH treatment. This may be connected with energy-dependent processes for iodide trapping or with an energy requirement for engulfment of colloid. Thyrotropin also causes a 2- to 3-fold stimulation of ^{32}P uptake into total lipid phosphorus in various thyroid tissues (Freinkel, 1957). Both Freinkel (1957) and Vilkki (1961) noted that the most prominent labelling was in the phosphoinositide fraction under in vitro conditions. An enhancing effect of TSH upon the incorporation of (^{14}C) inositol into phosphatide has also been reported (Freinkel, 1960). These latter observations suggest that there may be an in vitro renewal of the entire phosphoinositide

molecule following TSH administration.

(e) Effects on Iodine Metabolism TSH promptly enhances glandular production and release of iodide, and the stimulation of glandular iodide production includes not only an increased rate of liberation of iodotyrosines from peptide linkage, but also an increased deiodinating activity (Nagataki, Shizume and Okinaka, 1961; Rosenberg, Athans, Ahn, and Behar, 1961).

Tong (1964) has also demonstrated a stimulatory effect of TSH on the synthesis of thyroxine by isolated thyroid cells. The effect of thyrotropin on the uptake of iodine appears to occur at a later time. Halmi and co-workers (Halmi, 1957; Halmi, Granner, Doughman, Peters and Müller, 1960) have presented detailed evidence that the iodide 'trap' in the rat thyroid gland is reduced shortly after TSH administration and values above normal are not obtained until the later phases of TSH action.

This is due to an initial enhanced exit of thyroidal iodide, which explains the biphasic response (Halmi et al., 1960; Halmi, 1961).

(f) Effects on Amino Acid and Protein Metabolism Proteolysis of thyroglobulin occurs virtually immediately on TSH treatment. Colloid droplets appear to be engulfed from the lumen into the

thyroid cells and their thyroglobulin content degraded by lysosomal proteolytic enzymes with the release of thyroid hormone (Wollman, Spicer and Burstone, 1964). Debons and Pitman (1962) have demonstrated a TSH-enhanced uptake of amino-isobutyric acid into thyroid gland slices in vitro. Raghupathy, Tong and Chaikoff (1963) have shown that there is an increase in amino acid uptake into the protein of tissue slices from TSH-treated animals and TSH also stimulates the incorporation of (^{14}C) leucine and ^{131}I into protein by isolated thyroid cells (Tong, 1965; Raghupathy, Kerkof and Chaikoff, 1965).

(g) Effects on Nucleic Acid Metabolism Fiala, Sproul and Fiala (1957) demonstrated that TSH administered in vivo increased the RNA content of rat thyroid homogenates. It was subsequently shown by Matovinovic and Vickery (1959) and Ekholm and Pantić (1963) that prolonged treatment with thyrotropin in vivo caused an enhancement in the RNA content of the thyroid followed by an increase in DNA content due to cell division. These effects were accompanied by an increase in thyroid protein content. Hall (1963, 1965) has shown that TSH increased the uptake of (^{14}C) formate into the purines of tissue slice RNA after a 3-hour

incubation period. He attributed this effect to an increase in available ribose for purine synthesis caused by TSH stimulation of glucose oxidation via the hexose monophosphate pathway. Hall (1963) also showed that (^{14}C) adenine incorporation into the RNA of calf-thyroid slices was raised by TSH. This suggests that TSH may also increase the uptake of labelled precursors into RNA by stimulating RNA turnover as well as having an effect on the rate of synthesis of the precursors.

Thus it can be seen that once the full metabolic response to thyrotropin has been established all phases of thyroid metabolic turnover are activated. However from current knowledge it is impossible to determine the sequence of these TSH-stimulated events, and thus no single step can as yet be defined as the trigger mechanism in response to TSH, if indeed such a single mechanism exists. The evidence of relationships between the various actions of TSH on the thyroid gland will be reconsidered after our own experiments have been described.

From the experimental evidence described above there is little information concerning the early actions of TSH on the nucleic acid metabolism of the thyroid follicular cells. Hormones

act directly or indirectly by controlling the activity of genes which are in a continual state of flux in response to particular stimuli. It is currently thought that the site of action of many hormones is at or near the genetic level on the basis of studies of hormonal effects on the control of cellular protein synthesis. The current concepts of the processes involved in the regulation of cellular protein biosynthesis will be described briefly in order to facilitate discussion of reported hormonal effects on these regulatory mechanisms.

Control Mechanisms of Protein Synthesis

Any theories on the regulation of the biochemical reactions involved in cellular protein synthesis must be considered in the light of present knowledge of the mechanisms involved in these synthetic processes. The general process of information transfer from gene to protein has been resolved into two stages, namely transcription and translation.

Transcription Process

There is substantial evidence that the genetic code is contained in the sequence of nucleotides of the DNA molecule (Crick, 1963). It appears that the expression of genetic function

is first achieved by the synthesis of RNA molecules, the nucleotide sequences of which are prescribed by DNA. Some of this RNA, mRNA, is thought to direct the synthesis of proteins. There is a great deal of evidence to show that each amino acid corresponds to certain triplet sequences of nucleotides (codons) on the mRNA, and that sequences of amino acids in the protein molecules are determined by sequences of codons in the mRNA (Crick, 1963; Lipmann, 1963). However it has been reported by several workers that the code is degenerate (Crick, Barnett, Brenner and Watts-Tobin, 1961; Weisblum, Benzer and Holley, 1962). The genetic information coded in the mRNA is transferred to the cytoplasmic sites of protein synthesis.

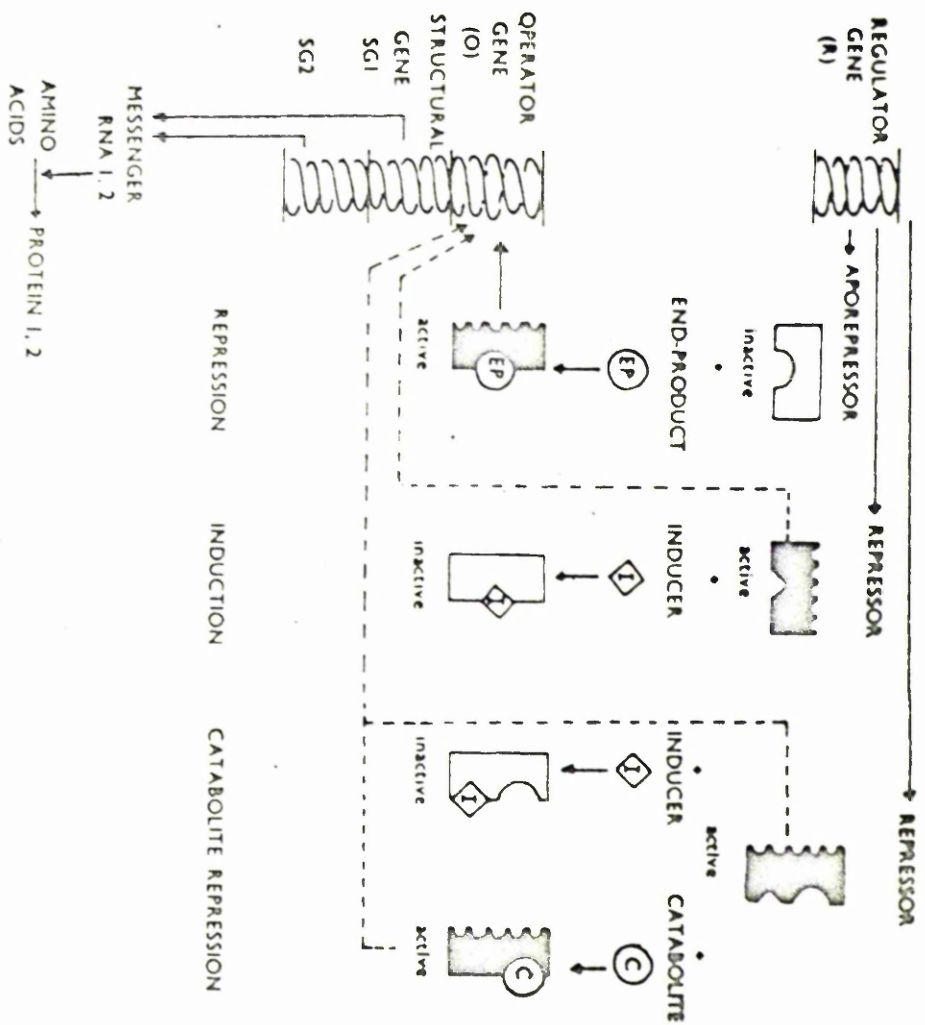
Translation Process

Ribonucleoprotein particles (ribosomes) become attached to the mRNA forming polysomes which are believed to be the structures normally engaged in protein synthesis (Wettstein, Staehelin and Noll, 1963). The nature of the processes involved in polysome formation are not yet clear, but it appears that smaller ribosomal sub-units become attached

to the mRNA strand, and this is followed by addition of the larger ribosomal sub-units, thereby initiating polysome formation (Joklik and Becker, 1965a, 1965b; Henshaw, Revel and Hiatt, 1965; Girard, Latham, Penman and Darnell, 1965). The site of polysome formation has not been located, but there is some evidence for nucleolar involvement in this process (Desjardins, Smetana, Steele and Busch, 1963; Birnsteil and Hyde, 1963; Hurlbert, Liau and Orengo, 1964). Amino acids bound to their specific transfer RNA (tRNA) molecules are then assembled in their correct order on the polysome by means of an anticodon to mRNA in the nucleotide sequence of the tRNA molecules. Polypeptide formation then takes place starting from the N-terminal end (Bishop, Leahy and Schweet, 1960).

Theories of Jacob and Monod

At the moment the most important line of research into the control of protein synthesis has come from the studies of Jacob, Monod and their co-workers on the control of enzyme induction and repression in bacterial systems (Jacob and Monod, 1961; Monod, Changeux and Jacob, 1963). An outline of their proposals for the genetic control of protein synthesis in bacteria



Scheme for the genetic control of protein synthesis in bacteria (based on Monod, Chazotte & Jacob (1963) and Jacob & Monod (1961)). The functional unit, the operon, equis the operator gene (O), plus the structural gene(s) (SG) it regulates. The cytoplasmic repressor substance produced by the regulator gene is shown in the active form (i.e. inhibits the operator) by solid symbols and in the inactive form by open symbols. Suggested mechanisms to account for repression, feedback inhibition and katabolite repression are shown.

Fig. 4 (From Fry, 1964).

is shown in Fig. 4.

The transcription process is controlled at the level of messenger synthesis by specific agents, the repressors, able to recognise and bind selectively certain genetic loci, called operators, which apparently function as exclusive initiation points for the first transcription. The DNA segment whose transcription is thus 'co-ordinated' by a given operator may involve one or several genes or cistrons; it constitutes an 'operon'. The synthesis of the corresponding protein(s) is therefore governed by the homologous repressor which in turn is synthesised under the control of a specialized 'regulator' gene. In most, if not all cases, the activity of the repressor is controlled by specific molecular compounds acting either as positive effectors (activating the repressor, and thereby blocking messenger and protein synthesis), or as 'inducers' (inhibiting the repressor, and thereby inducing the synthesis of messenger and of proteins).

Jacob and Monod (1961) originally suggested that the repressor was a polyribonucleotide, but this assumption cannot by itself account for repressor-inducer interaction (Jacob,

Sussman and Monod, 1962). Several indirect lines of evidence suggest that the active product of a regulator gene is a protein present in exceedingly small amounts in cells (Monod et al, 1963; Garen and Garen, 1963). However, no protein with this specific property has been isolated as yet.

Monod et al.(1963) also suggest that hormones could act as allosteric effectors, each of them able specifically to trigger allosteric transitions in a variety of proteins, mostly enzymes but also genetic repressors, by producing reversible conformational alterations in the proteins on binding. So far there is no direct evidence that hormones act in this way in the living cell; however it has been shown that thyroxine can cause conformational alterations of this type in the isolated enzyme glutamic dehydrogenase (Wolff, 1964b).

The proposals of Jacob and Monod, however, leave certain questions unanswered. For example, does the repressor operate at the gene, or at the mRNA level or at both, and if repressors are proteins are they subject to the same type of control mechanism? Yanagisawa (1963) has suggested that the repressor may act at both gene and

messenger levels. From experiments of Geiduschek, Nakamoto and Weiss (1961) and Wood and Berg (1962) it has been proposed that native DNA serves as a template for RNA synthesis via the following steps:- (a) local unwinding of DNA, (b) synthesis of RNA on one strand of DNA as template, and (c) release of RNA from the DNA by rewinding of the DNA double helix. These steps suggest three sites of action for the hypothetical repressor molecule; either the DNA strand that serves as template for RNA synthesis, the DNA strand that serves as release factor, or the RNA product itself. Frenster (1965) has proposed that one strand of the uncoiled DNA serves as template for RNA synthesis, while the other acts as a binding site for specific derepressor molecules.

It is difficult at present, however, to decide how far theories of control mechanisms in bacterial systems can be extended to mammalian systems. The extensive and rapid turnover of mammalian proteins as opposed to bacterial proteins introduces further complications, and any theory of control of mammalian protein metabolism must include not only regulatory mechanisms for protein synthesis but also for

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protein degradation, since the level of a tissue protein or enzyme at any one time reflects an equilibrium between these two processes. Further, the chemical composition and structure of the genetic apparatus in mammalian cells is different from that in bacteria, especially in the amount of DNA-associated histone in the former.

Role of the Histones

It has been suggested that histones and possibly other nuclear proteins repress gene activity (Stedman and Stedman, 1950). The possibility that they serve as regulators of chromosomal activity by interacting with specific segments of DNA, thereby preventing the transcription of genetic messenger from that locus, has attracted considerable interest. Work in several laboratories has demonstrated that histones are indeed potent inhibitors of the DNA-dependent synthesis of RNA in vitro (Huang and Bonner, 1962; Hindley, 1963; Allfrey and Mirsky, 1963). In addition, it has been shown that removal of histones from chromatin increased its RNA-synthesising capacity (Huang and Bonner, 1962) and that pre-incubation of calf-thymus

nuclei with trypsin to remove most of the histones caused the synthesis of RNA to be substantially augmented (Allfrey, Littau and Mirsky, 1963). These reports establish histones as general inhibitors of the DNA-dependent RNA polymerase system but do not in themselves establish that histones function in a selective way in regulating the transcription process in vivo. In addition, this effect of histones is not specific since added histones inhibit many other synthetic reactions in nuclei, as well as some occurring in mitochondria and isolated enzyme systems.

There is as yet no evidence of hormone action on histone synthesis and activity although binding of hormones to histones in vivo has been reported. Sekeris and Lang (1965) have shown that, during the first few hours after administration of (^3H) cortisone, a considerable part of the radioactivity is bound to the histones of rat-liver nuclei. Also it has been reported by Wilson and Loeb (1965) in a study of the localization of (^3H) testosterone in a target organ, that there was considerable binding of the testosterone to the histone of the euchromatin fraction of the genetic apparatus. Dahmus and Bonner (1965)

have shown that chromatin isolated from livers of cortisol-treated adrenalectomized rats possessed a greater template activity for DNA-dependent RNA synthesis than did chromatin isolated from control rats, and that this difference in template activity was abolished by removal of proteins associated with the DNA.

Specific Inhibitors of Protein Synthesis

Recently some antibiotics, which are known to inhibit certain stages in protein synthesis, have been used to try and locate the site of action of hormones on this process.

Actinomycin D and puromycin have been used most extensively in the studies of this nature.

Actinomycin D: This inhibits the transcription process by combining with the deoxyguanosine residues of DNA preventing DNA-dependent synthesis of RNA (Reich, 1963).

Puromycin: This inhibits protein synthesis at the stage of polypeptide chain growth by substituting for the incoming acyl-sRNA complex; it is subsequently released with the incomplete peptide chain (Yarmolinsky and De la Haba, 1959; Allen and Zamecnik, 1962).

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The use of these antibiotics has indicated certain differences in the mechanism of hormone induction of protein synthesis at the transcriptional and translational levels, although the results must be interpreted with caution since these compounds are rather toxic and may have other effects both in vivo and in vitro (Acs, Reich and Valanju, 1963; Girard, Penman and Darnell, 1964; Honig and Rabinowitz, 1965; Greif, Song and Chipkin, 1965). Also, in studies using isotopic tracers as a measure of synthesis and turnover of nucleic acids and protein, consideration must be taken of the time relationships of administration of antibiotic, hormone and isotope before any conclusions may be drawn concerning hormone action.

Site of Action of Hormones on
Protein Biosynthetic Mechanisms

Many hormones stimulate the biosynthesis of enzymes and of proteins in general (see Karlson, 1965 for a review). It has been shown that puromycin inhibits glucocorticoid induction in the livers of adrenalectomized rats of several enzymes, e.g. tryptophan pyrrolase (Goldstein, Stella and Knox, 1962), and tyrosine- α -ketoglutarate transaminase (Garren, Howell,

Tomkins and Crocco, 1964). It was subsequently found that glucocorticoid-induced increases in these enzymes were preceded by an initial activation of RNA synthesis and that actinomycin D prevented this hormonal action (Garren et al, 1964; Barnabei, Romano and Di Bitonto, 1965). Barnabei et al. (1965) also demonstrated an increase in DNA-dependent RNA polymerase activity at short time intervals after glucocorticoid administration.

From other studies on enzyme induction by certain hormones using actinomycin D and puromycin, e.g. oestrogen (Hamilton, 1963), testosterone (Williams-Ashman, Liao, Hancock, Jurkowitz and Silverman, 1964), and aldosterone (Porter, Bogoroch and Edelman, 1964), it appears possible that the primary effect of these hormones on their target organs could be due to synthesis of additional RNA (mRNA). Indeed, in many cases, an acceleration of synthesis of rapidly-labelled nuclear RNA or a stimulation of DNA-dependent RNA polymerase activity in nuclear preparations has been demonstrated (Talwar, Gupta and Gros, 1964; Pegg and Korner, 1965; Sekeris, 1965; Williams-Ashman, 1965; Gorski, Noteboom and Nicolette, 1965; Tata and Widnell, 1966). This is also supported by work of Kidson and Kirby (1964) showing

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selective increases in rapidly labelled fractions of liver-cell RNA by a variety of hormones, including insulin and thyroxine. Therefore much evidence is now available showing that many growth-promoting and developmental hormones regulate the RNA and protein synthesis of their target cells.

In order to determine whether some common mechanisms or even sites are involved in the regulation of RNA synthesis by different hormones, Widnell and Tata (1966) have examined RNA synthesis in rat liver which depends on more than one hormone for its normal function. They studied its response to growth hormone, triiodothyronine and testosterone. It was found that these hormones do not compete with each other in regulating RNA synthesis; all the evidence supports the idea that their actions on RNA metabolism are mediated via different routes. There have also been claims that RNA, extracted from tissues of hormone-treated animals, can mimic the effects produced by the hormone when applied to tissues of control animals or can stimulate enzyme synthesis when added to cell-free protein-synthesising systems (Segal, Davidson and Wada, 1965; Sekeris and Lang, 1964).

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All this makes a formidable case of the general hypothesis that hormones regulate protein synthesis and control metabolic pathways by direct derepression of selected regions of the genome so that new specific messenger-RNA molecules are produced which in turn produce more molecules of particular cell proteins, some of them enzymes. This is the hypothesis proposed by Karlson (1963), namely that many hormones alter the rate of transcription of specific genes during hormonal induction of enzymes. This conclusion was reached from studies which Karlson and his co-workers had carried out on the induction of the enzyme dopa decarboxylase in the larval forms of *Chironimus* and *Calliphora* by the insect metamorphosis hormone, ecdysone. This hormone produced regions of active RNA synthesis at certain loci on the giant chromosomes of the *Chironimus* salivary gland, and was also found to react with the epidermal-cell nuclei of *Calliphora* larvae (Karlson, Sekeris and Maurer, 1964). Further, the RNA produced in the epidermis of *Calliphora* larvae under the influence of ecdysone was found to stimulate the synthesis of dopa decarboxylase in a cell-free protein-synthesising system prepared from rat liver (Sekeris

and Lang, 1964).

However, several pieces of evidence do not fit into this hypothesis of hormonal action on the transcription process. Insulin, for example, continues to stimulate glucose and amino acid uptake by isolated rat diaphragms in the presence of amounts of actinomycin D sufficient to stop RNA synthesis (Eboué-Bonis, Chambaut, Volfin and Clauser, 1963; Wool and Moyer, 1964). Ray, Forster and Lardy (1964) found cortisol stimulation of gluconeogenesis whether rats had been treated with actinomycin D or not, and Lippe and Szego (1965) argue that much of the actinomycin D action in inhibiting the actions of oestrogens in the uterus can be explained by elevated blood levels of corticosteroids caused by these hormones. Also the effect of TSH on thyroid glucose oxidation, which has been mentioned earlier, still occurs in the presence of doses of actinomycin D and puromycin which are effective in inhibiting RNA and protein synthesis (Field, Johnson, Kendig and Pastan, 1963; Field, Epstein and Jarrett, 1965). Korner (1965a) noted that the number of ribosomes and their ability to attach polyuridylic acid was altered by changes in growth hormone level; factors other than

mRNA are thus involved in the growth hormone action.

There is also growing genetic evidence, from studies in bacterial systems, of the existence of control of protein synthesis at the translational level stage of message to protein as well as the transcription stage of gene to message (Beckwith, 1964; Brenner, Stetton and Kaplan, 1965). Garren and co-workers (Garren, Howell, Tomkins and Crocco, 1964; Garren, Ney and Davis, 1965) have presented some evidence that hormone-induced enzyme synthesis may in some cases be controlled at the translational level. This conclusion was drawn from time-course studies of the effects of actinomycin D and puromycin on (a) cortisol induction of certain enzymes in the livers of adrenalectomized rats (Garren et al, 1964), and (b) adrenocorticotropin effects on corticosterone production in the rat adrenal gland (Garren et al, 1965). There is further evidence that glucocorticoid induction of liver enzymes may be controlled at the translational level as well as at the transcriptional level. Kenney and Albritton (1965) studied the effects of actinomycin D on the induction of liver tyrosine transaminase of adrenalectomized rats and on the repression of synthesis of this enzyme by tyrosine. They found

that actinomycin D does not by itself influence the enzyme level but blocks the tyrosine-initiated repression of enzyme synthesis, indicating that repression acts at the translational level whereas the initiation of repression involves transcriptional processes. In agreement with the above evidence for control of hormone action at the cytoplasmic level, Hamilton (1964) found that, during the response of the uterine cells to oestrogen, changes in protein synthesis occurred which were not actinomycin D-sensitive and which preceded any demonstrable effects on RNA synthesis.

Therefore a demonstration that mRNA synthesis is enhanced by hormone treatment does not prove that this is the point of action of the hormone, for messenger synthesis may be a secondary result of enhancement of protein synthesis by other means, such as an alteration of the enzyme-forming systems or a change in activities of the enzyme proteins themselves.

However, there is further evidence that the site of action of some hormones is nuclear if not directly at the genetic level. Certain hormones, after in vivo administration, have

been localized predominantly in the nuclei of their target organs. (^3H) aldosterone is located in the nuclei of toad-bladder cells when affecting sodium transport, whereas (^3H) progesterone under the same conditions is not (Porter, Bogoroch and Edelman, 1964). It has been shown, as described earlier, that, after administration of radioactive testosterone and cortisone, a considerable part of the radioactivity was located bound to nucleo-histone fractions of their target organs (Wilson and Loeb, 1965; Sekeris and Lang, 1965). Also, using a fluorescent antibody technique, Greenspan and Hargadine (1965) have located TSH in the nuclei of guinea pig- and dog-thyroid epithelia after hormone administration. However, for detection purposes, the amounts of hormone used in these experiments were much greater than the normal physiological levels with the result that the target-organ cells may not have been able to cope with the large influx of hormone and non-specific binding of the hormone to some cellular components may have taken place. Intranuclear localization of hormones does not however seem to be general. Dingman and Sporn (1965) have demonstrated from studies on cellular

localization of actinomycin D and cortisol, that actinomycin D was found to be bound to nuclear structures whereas cortisol was not so bound under the same experimental conditions. On the other hand, Kenney and Kull (1963) observed that shortly after cortisol administration to rats there was a rapid increase in labelling of the RNA of the liver nuclei. Thus, as mentioned earlier, the appearance of rapidly-labelled nuclear RNA in a target organ after hormone administration does not necessarily indicate that the site of hormonal action is at the genetic level.

There is no positive evidence as yet for hormones having effects on isolated nuclei of their target organs in vitro apart from reports of direct actions of ecdysone on the RNA metabolism of isolated epidermal-cell nuclei of *Calliphora* larvae (Sekeris, Dukes and Schmid, 1965) and of cortisol on the RNA metabolism of rat-liver nuclei (Dukes and Sekeris, 1965). Thus, in spite of the recent advances in the elucidation of the mechanisms involved in protein synthesis, there is no proof as yet of a direct hormonal action at the molecular level, although it would appear from experimental evidence that different hormones may have different cellular sites of action thereby influencing protein synthetic

mechanisms at different levels.

Nature of the Present Investigations

The nature of the present work was designed to investigate the effects of the hormone, thyrotropin, on the synthesis of nucleic acid and protein in the thyroid gland in order to find a specific mode of action of the hormone on these processes. Preliminary investigations were carried out to devise means of estimating the RNA and DNA of the thyroid gland. A study was then made of the nucleic acid, protein and phospholipid content of the thyroid glands of different mammalian species, and relationships to thyroid weight and total body weight established.

Isotopic studies were then carried out using sheep-thyroid gland slices to study the effects of TSH on RNA metabolism. The effects of actinomycin D and puromycin were also studied in this system. On preparing nuclear and cytoplasmic fractions from the slices after incubation with hormone and a radioactive RNA precursor, it was found that TSH had a greater effect on the nuclear RNA fraction and that this occurred at earlier time intervals than in the case of the cytoplasmic RNA fraction.

The action of TSH on the incorporation of isotopic precursors into the RNA and protein of isolated sheep-thyroid nuclei was then investigated. The effects of actinomycin D and puromycin on this process were again examined. Finally studies were carried out on DNA-dependent RNA polymerase fractions prepared from sheep-thyroid nuclei and on the changes they undergo after treatment of the nuclei with TSH. From these observations some conclusions about the mode of action of TSH have been deduced.

SECTION I

- (a) Investigations into methods of estimating thyroid nucleic acids.
- (b) Studies on the protein, nucleic acid, and phospholipid content of the thyroid glands of different mammals.

INTRODUCTION

As a preliminary to examining control of protein biosynthesis in the thyroid gland, a study was made of the composition of the thyroid glands from a range of mammals. Particular emphasis was placed on the nucleic acid and protein content of the gland, since nucleic acids are intimately involved in the protein synthesis mechanism. Although the literature already contains some estimates of the concentrations of nucleic acids in the thyroids of several species, the considerable errors inherent in most methods of nucleic acid analysis (Hutchison and Munro, 1961; Munro and Fleck, 1966), invalidate comparisons between the published data for different species.

The problem of nucleic acid analysis in tissues has been reviewed ^{by} Hutchison and Munro (1961), and Munro and Fleck (1966). From these reviews, it appears that the most suitable method of estimating the nucleic acid content of animal tissues is that of Schmidt and Thannhauser (1945). In this procedure the tissue RNA is hydrolysed by alkali to fragments no longer precipitable upon acidification, whereas the DNA of the tissue resists attack

by alkali and can be reprecipitated upon acidification.

Thereafter the RNA of the acid-soluble fraction can be estimated in one of three ways, either by phosphorus estimation, by its ribose content or by means of its strong ultraviolet absorption at 260m μ . However each of these methods of assay is liable to errors arising from contamination of the RNA fraction with other tissue constituents (Hutchison and Munro, 1961). The DNA content of the acid-precipitable fraction can be estimated by measurement of deoxyribose content or phosphorus content.

Fleck and Munro (1962) explored the use of ultraviolet absorption measurements for RNA estimation and found that the usual prolonged alkaline digestion of rat liver samples releases a considerable amount of tissue protein in an acid-soluble form which seriously increases the ultraviolet absorption of the RNA fraction of the digest. However, if hydrolysis is carried out for only a short time in dilute alkali, all the liver RNA is released in acid-soluble form without significant contamination by polypeptide material, and thus an accurate estimate of rat-liver RNA can be obtained from the extinction of the acid-soluble fraction at 260m μ .

However when measurements of thyroid RNA content were carried out using this short-digestion form of the Schmidt-Thannhauser procedure, the acid-soluble RNA fractions obtained were found to be contaminated to a considerable extent with polypeptide material absorbing at 260m μ , and thus accurate estimates of thyroid RNA content could not be made by direct measurement of the ultraviolet absorption at 260m μ . The polypeptide material responsible for this contamination of the RNA fraction arose from extensive breakdown of thyroglobulin resulting in alkaline degradation products which were acid-soluble. Thus correction procedures had to be devised in order to obtain quantitative estimates of thyroid RNA. Thyroglobulin was also found to interfere with the estimation of DNA by colorimetric measurement of deoxyribose, and again investigations had to be carried out to find an accurate method for assaying thyroid DNA.

When standard procedures had been devised for the special case of the thyroid gland, studies on the nucleic acid content of the thyroid glands of various species were carried out with a view to establishing a relationship between the

composition of the various components of the gland and species characteristics such as body weight and thyroid follicle size.

The reason for exploring such relationships is as follows:

Basal energy metabolism is related to the 0.73 power of body weight (Brody, 1945), and thus the intensity of the metabolism of mammals diminishes with increasing size of species. A similar relationship to body weight ($W^{0.7}$) is followed by several parameters of mammalian protein metabolism, such as the rate of exchange of (^{15}N)-labelled amino acids with tissue proteins and the turnover of plasma albumin (Munro, 1964). It might be anticipated that this effect of body size on the intensity of protein synthesis would be reflected in differences in the concentration of tissue nucleic acids, since they are concerned with protein synthesis.

This hypothesis was tested by Munro and Downie (1964) who were able to show that, as the size of the species of mammal increases, the amount of RNA per liver cell progressively decreases. This effect of body size on liver cell composition could be correlated with a slower rate of synthesis of plasma proteins in the larger animals.

Thus studies were carried out on thyroid cell

composition to see if a similar relationship exists between thyroid composition and the animal size.

MATERIALS AND METHODS

Tissue Preparation

Thyroid tissue was obtained from rats, rabbits, cats, dogs, sheep, pigs and cows. Healthy adult animals on their usual diets were used; they were all males except in the case of cows and sheep (lambs). The thyroid glands were excised as quickly as possible after killing and the capsules and all extraneous tissue removed. The glands from rats were first frozen to prevent loss of colloid while adherent fibrous tissue was being removed. The glands were then analysed individually, except for the rats where the glands of several animals were pooled. The material from rats and rabbits was carefully checked histologically to ensure the absence of tissues other than thyroid. One sample of histologically normal human thyroid was also analysed; it was obtained from an adult man during operative removal of a benign tumour in the thyroid region.

Tissue Analysis

Thyroid tissue was homogenized in 19 volumes ice-cold water using a Nelco Blender except in the case of rats and

Fig. 5.

SEPARATION OF NUCLEIC ACIDS (Fleck and Munro, 1962)

1 in 20 homogenate of thyroid tissue in water at 0° prepared using Nelco Blender.

5ml samples of a 1 in 20 homogenate

Add 25ml 0.6N-HClO₄. Stand 10 min. in ice, then centrifuge at 1,000g for 10 min. at 0°.

↓
Supernatant

(discard)

↓
Precipitate

Wash twice with 5ml 0.2N-HClO₄ discarding the washings. Ppt. drained, and 4ml 0.3N-KOH added. Incubate at 37° for 1 hr. then cool in ice. Add 2.5ml 1.2N-HClO₄, and stand for 10 min. in ice. Centrifuge at 1,000g for 10 min.

↓
Precipitate

Wash twice with 5ml 0.2N-HClO₄ adding washings to the supernatant fraction.

↓
Precipitate (DNA fraction)

Dissolve in 0.3N-NaOH and make up to a suitable volume in 0.1N-NaOH for deoxyribose estimations.

↓
Combined Supernatant (RNA fraction)

Make up to a suitable volume in 0.1N-HClO₄ for ultraviolet absorption measurements at 260mμ.

150
rabbits where 29 volumes of water were used.

Estimation of Protein Content

This was carried out using the method of Lowry, Rosebrough, Farr and Randall (1951) using bovine serum albumin as standard. Assays were carried out on 1ml. samples of thyroid homogenate diluted 1 in 40.

Separation of Nucleic Acids

This was carried out according to the method of Fleck and Munro (1962) which is an adaptation of the alkaline digestion procedure of Schmidt and Thannhauser (1945). An outline of the procedure is shown in Fig. 5.

Ice-cold 0.6N-HClO₄ (2.5ml) was added to 5ml. of a cold aqueous homogenate of thyroid tissue in a centrifuge tube. After mixing and standing 10 min. at 0°, the precipitate was centrifuged down and washed twice with 5ml. 0.2N-HClO₄. (The choice of this concentration of acid and the omission of lipid extraction was based on data of Hallinan, Fleck and Munro (1963), who showed that extraction with lipid solvents causes loss of RNA.) Excess acid was drained off, 4ml. 0.3N-KOH added and the mixture incubated at 37° for 1 hr.

to degrade the RNA but not the DNA. The alkaline solution was cooled in ice and 2.5ml. cold 1.2N-HClO₄ added. After allowing the precipitate of DNA and protein to flocculate, it was centrifuged down and washed twice with 5ml. cold 0.2N-HClO₄. Supernatant fluid and washings (the acid-soluble "RNA" fraction) were made up to a suitable volume in 0.1N-HClO₄ for ultraviolet absorption measurements. The precipitate was dissolved up in a suitable volume of 0.1N-NaOH for estimation of DNA content.

Methods used during Evaluation of Nucleic Acid Procedures

Isolation of Polypeptide Material from the acid-soluble "RNA" fraction

The preparation of the acid-soluble polypeptide material of rat liver has been previously described (Fleck and Munro, 1962); a similar procedure was adopted for calf thyroid. A single batch of thyroid tissue (16g.) was subjected to the procedure for estimation of RNA as described above, but using appropriately increased quantities of reagents. The incubation time in 0.3N-KOH was increased to 5hr. in order to degrade the RNA to diffusible products (Fleck and Munro, 1962). The acid-

soluble fraction obtained was dialysed for 5 days at 4° against several changes of water and freeze-dried to yield approximately 20mg. of acid-soluble material, the protein content of which, when estimated by the method of Lowry et al. (1951), was 97%.

Estimation of Polypeptide in the acid-soluble "RNA" fraction

Samples (1ml.) of the acid-soluble fraction (0.1N with respect to HClO_4) were neutralised by the addition of 0.1ml. 1.0N-NaOH, then the reagents added for the estimation of protein by the procedure of Lowry et al. (1951). The appropriate acid-soluble peptide material was used as standard.

Estimation of Ribose

This was measured by the orcinol procedure of Kerr and Seraidarian (1945), the period of heating in the reagent being extended to 30 min. (Forbes, Steele and Munro, 1964). The colour formed was compared with that from ribose, and was expressed as RNA phosphorus on the assumption that 10µg. ribose give the same colour as 4.13µg. RNA phosphorus (Forbes et al., 1964).

Preparation of Purified RNA

RNA was prepared by the phenol method of Eason, Cline and Smellie (1963). The preparation was found to contain less than 1% protein (Lowry et al., 1951), less than 1% DNA (Ceriotti, 1952) and no detectable contaminating carbohydrate.

Estimation of DNA

Attempts were made to estimate thyroid DNA by measurements of deoxyribose and DNA-phosphorus.

Colorimetric Measurement of Deoxyribose

(a) Method of Ceriotti (1952): The principle of this method is that deoxyribose alone, when heated in the presence of conc. HCl, gives a colour which is not chloroform-extractable unlike the other mammalian tissue colour complexes formed during the reaction.

The procedure used for the DNA fractions prepared as described previously was as follows:- To 2ml. aliquots of the DNA solutions, 1ml. 0.04% indole and 1ml. conc. HCl were added, and the solutions mixed. The samples were heated for 10 min. at 100°, and then cooled rapidly. They were then extracted.

three times with 4ml. portions of CHCl_3 , discarding the CHCl_3 layers. On the last extraction the solutions were centrifuged at 100g for 5 min. before removing the CHCl_3 layer. The extinctions at 490m μ of the aqueous layers were then measured, and the values obtained compared with that from a standard solution of purified calf-thymus DNA. In all cases the extinction at 490m μ of a reagent blank was subtracted from the sample values.

(b) Modification of the Ceriotti Procedure (Schmid, Schmid and Brodie, 1963): 1ml. DNA solution and 1ml. monochloroacetic acid (final pH 2.5) were heated for 80 min. at 90°. The solutions were cooled and 1ml. 0.6% indole and 1ml. 10N-HCl added, and the resulting solutions heated for a further 80 min. at 85° for colour development. The samples were again cooled, extracted with CHCl_3 , and their extinctions at 490m μ measured as described in (a).

(c) Estimations of deoxypentose were also carried out using the diphenylamine reaction according to the modification of Burton (1956).

Determination of DNA by Phosphorus Estimation

The preparation of the DNA fractions was essentially the same as that already described (Fleck and Munro, 1962) except that after initial HClO_4 precipitation and washing and before undergoing alkaline digestion, the samples were extracted with a series of lipid solvents in order to remove phospholipids. The precipitates were drained free of acid and extracted successively with the following solvents centrifuging at each stage: 5ml. 90% ethanol containing 2% sodium acetate, 5ml. 95% ethanol and chloroform (3:1), 5ml. 95% ethanol and diethylether (3:1) and finally 5ml. diethylether. The addition of 2% sodium acetate to the ethanol was to minimise loss of RNA, DNA and protein into the first lipid extract (Steele, Okamura and Busch, 1964). The tissue residue was then dissolved in 4ml. 0.3N-KOH and incubated for 1 hr. at 37° as before. Phosphorus estimations were then carried out on samples of the DNA fractions in 0.1N-NaOH according to the method of Allen (1940), scaled down to one fifth.

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Final Technique for the Estimation of the
Nucleic Acids of Thyroid Glands from
different species

RNA and DNA were separated using the method of Fleck and Munro (1962) described previously. Ultraviolet absorption measurements at 260m μ were then carried out on the acid-soluble "RNA" fractions. However polypeptide material absorbing at 260m μ was also found to be present in the RNA fraction, and thus measurements at 260m μ could not be used directly for estimation of the RNA content. As a result, the protein content of the acid-soluble fractions was routinely measured (Lowry et al., 1951) as described previously to correct for polypeptide interference since the specific extinction co-efficient of the contaminating polypeptide at 260m μ was known (Table 3). In addition, corrections were made for polypeptide absorption by taking ultraviolet absorption measurements at 232m μ as well as 260m μ and computing the RNA content from a two wavelength correction procedure which is described in detail later.

The DNA fractions were prepared as described

previously (Fleck and Munro, 1962), but incorporating the lipid extraction procedure mentioned above. Measurements of deoxyribose were then carried out using the method of Ceriotti (1952), and of total phosphorus by the method of Allen (1940).

Estimation of Phospholipid Content

Measurements were carried out in two ways. Lipids were extracted quantitatively from thyroid homogenates (1 in 3 with water) by the procedure of Folch, Lees and Sloane-Stanley, 1957), or by extraction with a series of lipid solvents during the preparation of the DNA fractions described previously. The latter method of lipid extraction was used in cases where the total amount of thyroid tissue available for analysis was very small. The total phosphorus content of the lipid extracts obtained was determined by the method of Allen (1940), and the value obtained multiplied by 22.8 to give the weight of phospholipid present (Artom and Fishman, 1943). It was found that both methods of extracting lipids gave essentially similar values for phospholipid content when applied to samples from the same thyroid gland.

RESULTS AND DISCUSSION

Investigations into Methods of Estimating Thyroid RNA

The most suitable method for the estimation of the nucleic acids of animal tissues seems to be that of Schmidt and Thannhauser (1945), in which the tissue RNA and DNA are separated by digestion in alkali which hydrolyses the RNA to acid-soluble fragments whereas the DNA and most of the cell-protein are reprecipitated upon acidification (Hutchison and Munro, 1961). The RNA of the acid-soluble fraction can then be measured in several ways, each of which is liable to errors arising from contamination of the acid-soluble fraction with other tissue constituents as mentioned previously. From studies of Fleck and Munro (1962) on the use of ultraviolet absorption measurements for RNA determination, it appears that the usual prolonged alkaline digestion of liver samples releases a considerable amount of tissue protein in an acid-soluble form which seriously increases the ultraviolet absorption of the RNA fraction of the digest. However if alkaline hydrolysis is carried out for only 1 hr. at 37° in 0.3N-KOH, all the liver RNA is released in acid-soluble form

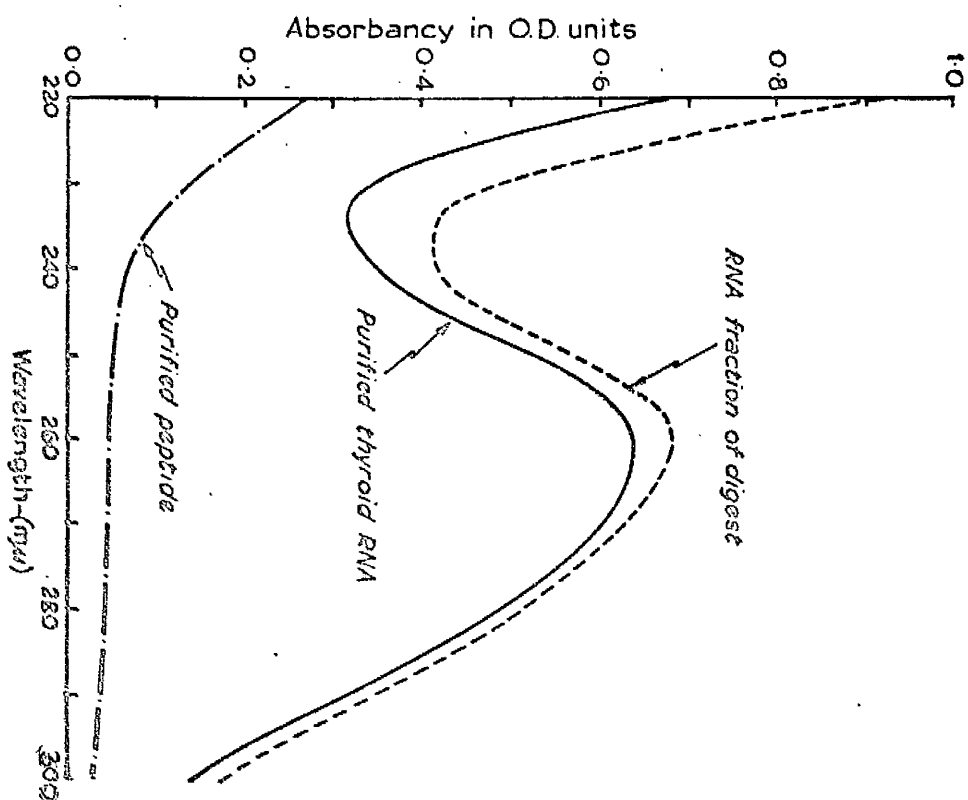
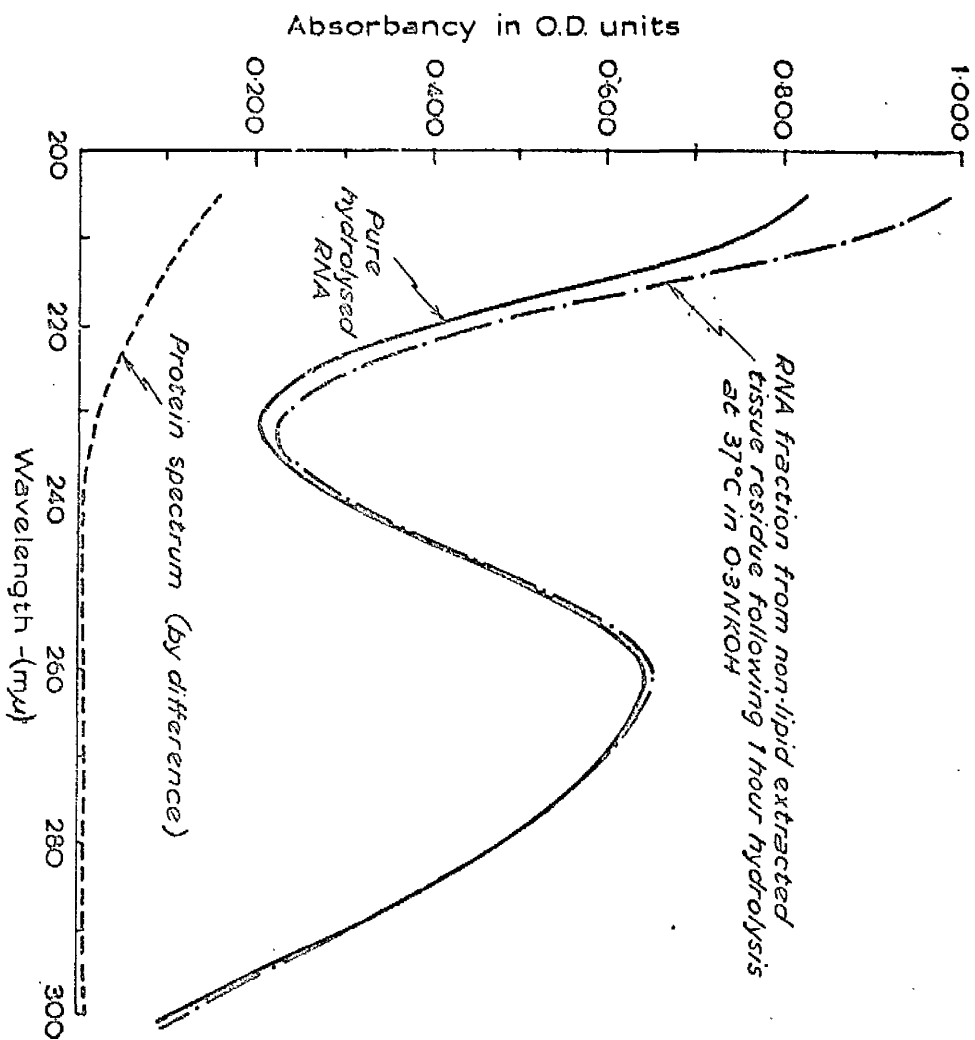
-57-

without significant contamination by polypeptide material, and thus an accurate estimate of rat-liver RNA content can be obtained from the extinction of the acid-soluble fraction at 260m μ (Fig. 6).

This method was applied to sheep, pig and calf-thyroid tissue and the ultraviolet absorption spectra of the acid-soluble "RNA" fractions in 0.1N-HClO₄ plotted over the range 220-300m μ (Fig. 6). When the spectra obtained were compared with that of a sample of purified RNA (Fig. 6), the absorption minimum at 232m μ characteristic of pure RNA is shifted to 240m μ in the tissue RNA fraction and the general level of ultraviolet absorption over this wavelength range is increased. These changes are suggestive of polypeptide contamination and this was confirmed by measurement of the protein content of the thyroid acid-soluble fractions (Lowry et al., 1951). It was also found that there was a greater degree of contamination the larger the species of animal thyroid used. This is reflected in the increase in thyroid protein content which is offset by a decrease in thyroid RNA content with increasing body size

Fig. 6

Absorption spectra of liver and thyroid RNA, acid-soluble "RNA" fractions and acid-soluble polypeptide materials. Each fraction was prepared as described in the text. The spectra were obtained using a recording spectrophotometer. All solutions were in 0.1N-HClO₄.



(Table 6). In different samples the contribution of peptide to the total ultraviolet absorption at 260m μ varied between 6 and 12%. This represents a much greater degree of polypeptide contamination in the acid-soluble fraction than was found by Fleck and Munro (1962) for liver samples, and presumably indicates that thyroid proteins are more readily hydrolysed to acid-soluble products by 0.3N-KOH. Thus direct measurement of thyroid RNA content by ultraviolet absorption at 260m μ of the acid-soluble fraction is unsatisfactory since the exact contribution of the interfering polypeptide material to the total absorption at 260m μ is unknown.

Estimates of the ribose content of the acid-soluble "RNA" fractions were then carried out using the orcinol procedure. Results were again unsatisfactory since the amount of polypeptide material present was sufficiently great to interfere in the colorimetric reaction. Interference could be due to both polypeptide and the carbohydrate residues of thyroglobulin, since both protein and sugars are known to interfere in the orcinol reaction (Hutchison and Munro, 1961).

Calf-thyroid samples were again subjected to the procedure

of Fleck and Munro (1962) for preparation of the acid-soluble "RNA" fractions but this time the samples were extracted with a series of lipid solvents before the alkaline digestion stage as described in the "methods" section for DNA. This was carried out to see if some of the contaminating peptide material could be removed on lipid extraction. However this procedure was found to increase further the amount of polypeptide subsequently appearing in the "RNA" fraction, as determined by the method of Lowry et al. (1951). The RNA fractions prepared without lipid extraction contained polypeptide at a concentration of $41\mu\text{g/ml}$, whereas the protein content of the lipid-extracted samples was about $70\mu\text{g/ml}$.

In order to check that acid-soluble products of thyroid protein were the source of contamination in thyroid "RNA" fractions, a sample of purified thyroglobulin (Derrien, Michel and Roche, 1948) obtained from Dr. Goudie (Western Infirmary, Glasgow) was subjected to the procedure used for nucleic acid separation, and the ultraviolet absorption spectrum of the resulting acid-soluble fraction plotted over the range 220-300m μ as shown in Fig. 7, and the protein content of the fraction was also measured (Lowry

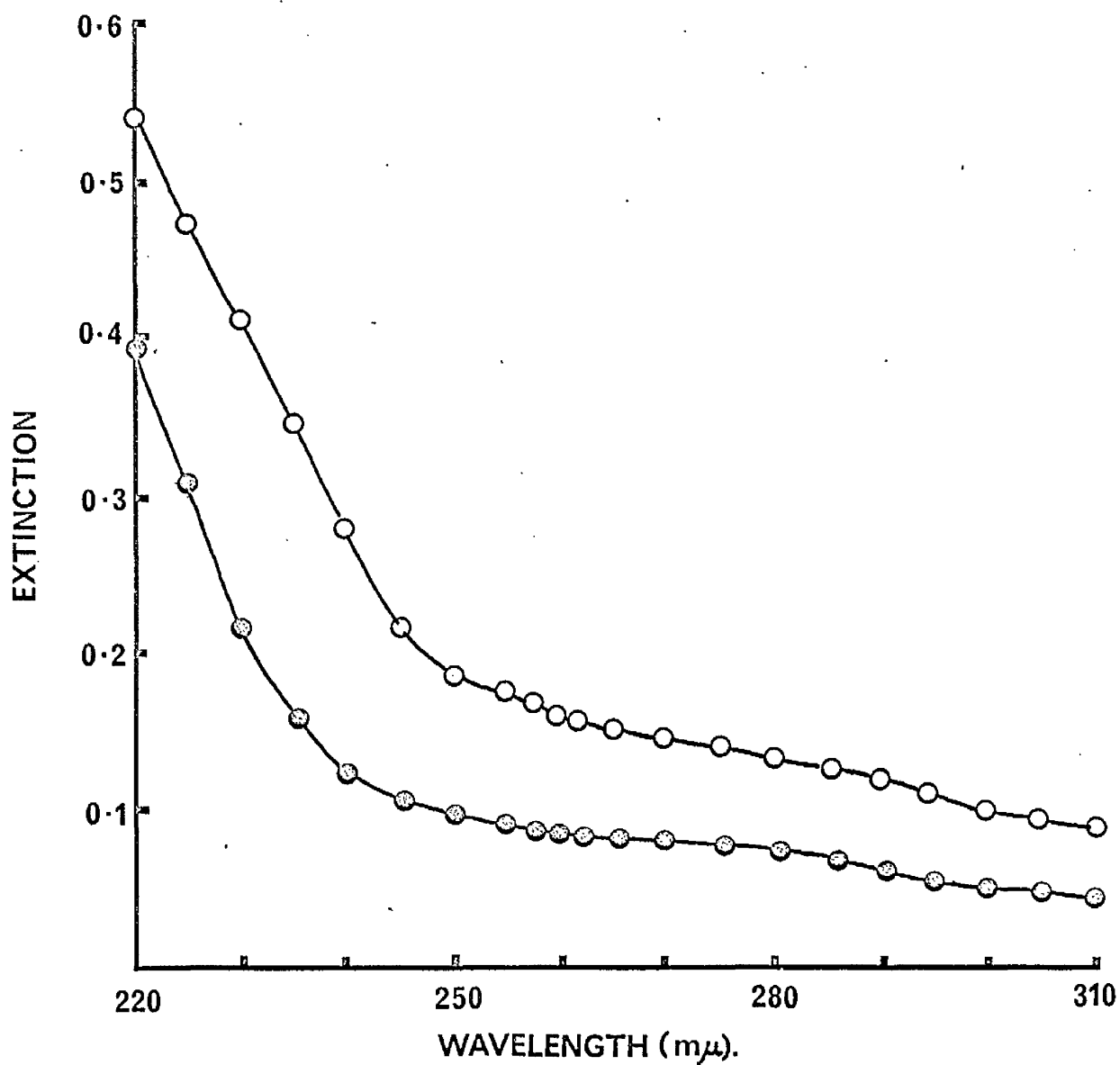


Fig. 7.

Comparison of the absorption spectrum of the contaminating polypeptide material (50 $\mu\text{g. protein/ml.}$), (\bullet), with that of the acid-soluble material from purified thyroglobulin (74 $\mu\text{g. protein/ml.}$), (\circ). Both samples were in 0.1N-HClO_4 .

et al., 1951). It can be seen from Fig. 7 that the ultraviolet absorption spectrum of the acid-soluble thyroglobulin fraction is very similar to that of the isolated contaminating polypeptide material, and the amount of thyroglobulin solubilized in acid (about 40%) was large. Thus acid-soluble degradation products of thyroglobulin are likely to be the source of polypeptide contamination in the acid-soluble "RNA" fraction. There is evidence that thyroglobulin is broken down by alkali into polypeptides which would probably be acid-soluble under the conditions used (Edelhoch, 1960).

Alkaline digestion studies on purified Calf-Thyroid RNA

Fleck and Munro (1962) treated samples of purified rat-liver RNA (Kirby, 1956) by digestion in 0.3N alkali for lengths of time varying between 15 min. and 24 hr. in order to study the release of RNA fragments into the acid-soluble fraction upon acidification. They found that the RNA was no longer precipitable with acid after 15 min. alkaline digestion, and in addition that the increment in ultraviolet absorption at 260m μ of the RNA fraction, produced as a result of alkaline hydrolysis, was fully developed within 1 hour. This increment in ultraviolet absorption i. e. the hyperchromic effect is due to hydrolysis of

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the RNA to smaller polyribonucleotides and finally to single nucleotides (Tsuboi, 1950). Fleck and Munro (1962) therefore selected an alkaline digestion period of 1 hr. for the separation of liver nucleic acids since after this time the hyperchromic effect was complete and also there was no significant release of polypeptide material into the acid-soluble RNA fraction at this time.

Similar investigations were carried out using purified calf-thyroid RNA. It was found from measurements of ribose content by the orcinol method that the RNA was no longer acid-precipitable after 30 min. alkaline digestion in 0.3N-KOH. A graph showing the changes in ultraviolet absorption at 260m μ of the RNA caused by alkaline digestion is shown in Fig. 8. From the graph it appears that the hyperchromic effect is not fully developed until after several hr. alkaline digestion, but is about 96% complete after 1 hr. This increment in ultraviolet absorption could not have been due to contaminating polypeptide material present in the RNA preparation becoming acid-soluble on prolonged alkaline digestion since the protein concentration of the preparation was less than 1% (Lowry et al.,

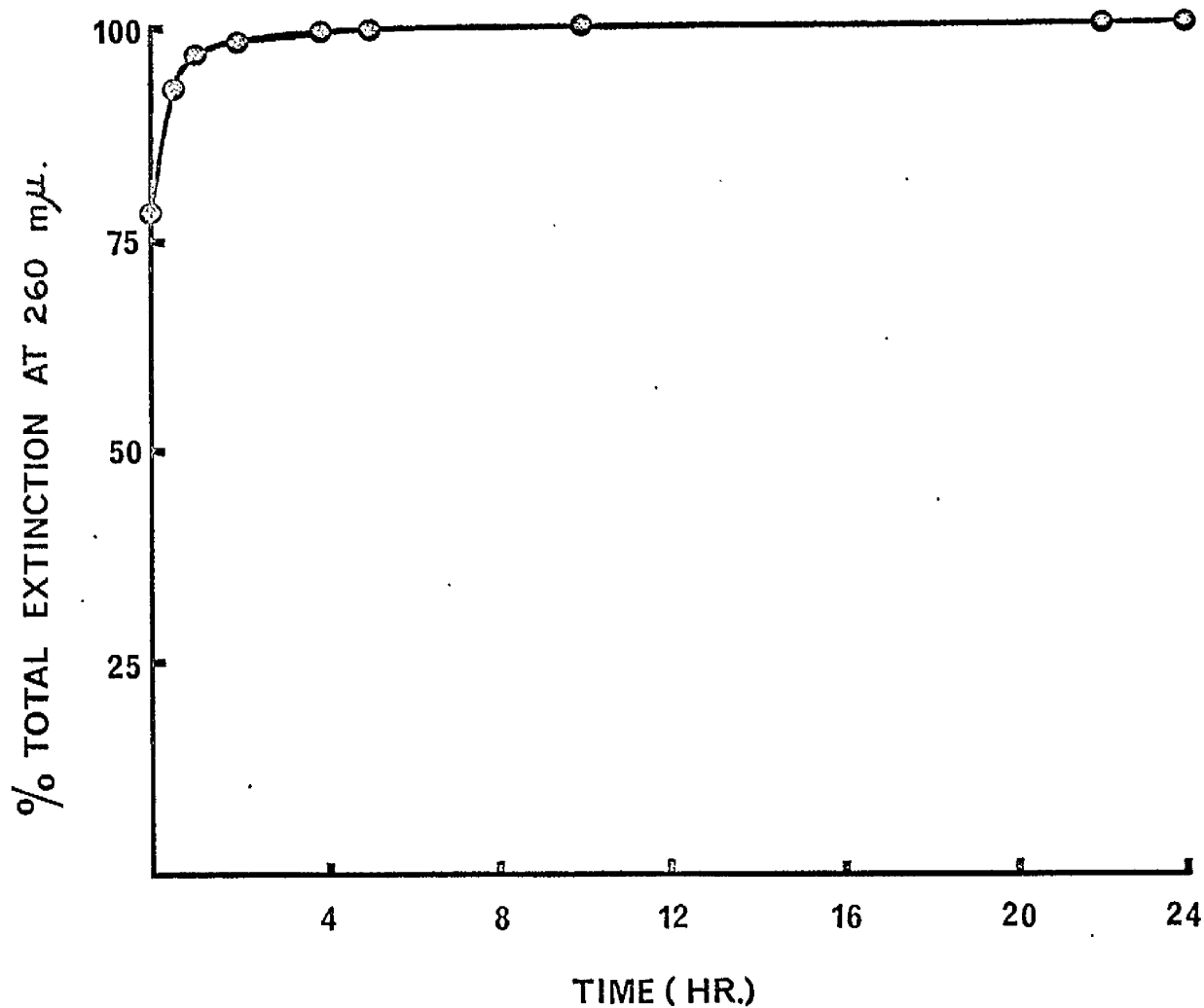


Fig. 8

Changes in the E_{260} value of purified calf-thyroid RNA caused by digestion at 37° in $0.3N$ -KOH. The digest was acidified to a final conc. of $0.1N$ - $HClO_4$ before the E_{260} measurements were made.

1951). It is also unlikely that the continued rise in absorption at 260m μ was due to deamination of cytidylic acid to uridylic acid since the concentration of alkali used was fairly low and rat-liver cytidylic acid was not deaminated under these conditions (Fleck and Munro, 1962). Therefore it would appear that the continued rise in ultraviolet absorption at 260m μ reflects a genuine hyperchromic effect which is not complete until about after 5 hr. alkaline digestion.

However it was decided to continue using an alkaline digestion period of 1 hr. since the hyperchromic effect was about 96% complete, and since prolonging the period of alkaline digestion with thyroid samples would seriously increase the amount of contaminating polypeptide material appearing in the RNA fraction, thus making estimates of RNA content by ultraviolet absorption measurements more difficult to correct for this source of error.

Corrections for the presence of Polypeptide in the acid-soluble RNA fraction

When samples of pure acid-soluble polypeptide material were available for utilization as standards, the

amount of polypeptide present in the acid-soluble "RNA" fractions could be estimated directly by the method of Lowry et al. (1951), and corrections made for the corresponding errors in ultraviolet absorption at 260m μ . (Tables 1 and 2). However this procedure is somewhat tedious with large numbers of samples and an ultraviolet spectrophotometric method of making this correction was considered, since "RNA" and polypeptide are the only two ultraviolet-absorbing substances present in detectable amounts in the acid-soluble fraction.

This necessitated the isolation of samples of purified thyroid RNA and of the contaminating polypeptide material for determination of their ultraviolet absorption characteristics. The results obtained for calf thyroid are shown in Fig. 6 and Table 3 where they are compared with data obtained for rat liver (Fleck and Munro, 1962; Fleck and Begg, 1965).

As mentioned previously, the ultraviolet absorption spectra of the acid-soluble "RNA" fractions from thyroid and liver when compared with those of the pure tissue RNA show the most obvious differences in absorption at 232m μ , the wavelength of minimal absorption of RNA (Fig. 6), and this

Table 1

The Estimation of RNA of Calf Thyroid

Sample	<u>Extinction</u>		Contribution to absorption at 260 mμ due to protein*	<u>Estimates of RNA (μg RNA-F/ml)</u>		
	at 232 mμ	at 260 mμ		a) from equation (1)	b) after correcting for protein	c) based on E ₂₆₀
1	0.270	0.480	0.024	1.42	1.38	1.45
2	0.182	0.210	0.034	0.51	0.53	0.63
3	0.435	0.625	0.060	1.73	1.71	1.89
4	0.374	0.478	0.063	1.25	1.26	1.44
5	0.530	0.641	0.100	1.64	1.61	1.94

* Determined by the method
of Lowry et al. (1951)

Mean
Differences
(b-a) = 0.01 < 1%
(c-b) = 0.17 ≈ 13%
(c-a) = 0.16 ≈ 12%

Table 2

The Estimation of RNA of Rat Liver

Sample	<u>Extinction</u>		Contribution to absorption at 260 mμ due to protein*	<u>Estimates of RNA</u> (μg RNA-P/ml)		
	at 232 mμ	at 260 mμ		a) from equation (2) for protein	b) after correcting for protein	c) based on E ₂₆₀
1	0.176	0.504	0.007	1.46	1.46	1.48
2	0.187	0.541	0.009	1.57	1.56	1.59
3	0.182	0.527	0.008	1.53	1.53	1.55
4	0.196	0.560	0.010	1.62	1.62	1.65
5	0.206	0.583	0.011	1.69	1.68	1.71
6	0.195	0.568	0.012	1.65	1.63	1.67

* Determined by the method of
Lowry et al. (1951)

Mean (a-b) = 0.01 ≈ 0.5%
Differences (c-b) = 0.03 ≈ 1.5%
(c-a) = 0.04 ≈ 2%

Table 3

Ultraviolet Absorption Data of Tissue RNA and
Acid-soluble Polypeptide Material

RNA		Absorption		$\frac{E_{232}}{E_{260}}$	$\frac{1\%}{1 \text{ cm}}$	
		wavelength	minimum		a) at 260 mμ	b) at 232 mμ
Acid-soluble polypeptide	1. Rat liver+ (Theoretical 10570)	9700	260	0.314	213	69.4
	2. Calf thyroid	9780	260	0.500	231	119
	1. Rat liver	—	275	255	2.36	10.3
	2. Calf thyroid				2.23	17.0
						43.0

* Molar extinction coefficient with respect to phosphorus.

+ The sample in fact contained 6.8% P, although the 'theoretical' P content of rat liver RNA is 9.7%.

The 'theoretical' figure was obtained by calculation from the base ratios quoted by Magasanik (1955).

is reflected in the corresponding increases in the $E_{232\text{m}\mu} / E_{260\text{m}\mu}$ ratios (Table 4). Thus in the acid-soluble "RNA" fractions from thyroid, the wavelength of minimal absorption had shifted from 232m μ to 240m μ and the absorption at 232m μ had increased by 31% whereas the contribution due to protein at 260m μ was only 11%. From Fig. 6 and Table 4 it can be seen that corrections for protein interference at 260m μ are necessary for accurate estimation of thyroid RNA content but not for rat-liver RNA content. A method of correction for protein was therefore devised on the basis of measurements at 232m μ and 260m μ . The method selected must be highly sensitive to changes in the polypeptide content of the "RNA" fraction owing to the relatively small amount of material extracted during the 1 hr. alkaline digestion period. Tsanev and Markov (1960) had devised a two wavelength correction procedure for rat liver using the method of Warburg and Christian (1942). However their method is unsuitable since they measured the contribution of the contaminating polypeptide in the region of 280m μ where the contribution due to polypeptide released during the short alkaline digestion period is too small

Table 4

Error in the Estimation of RNA due to
the Presence of Acid-Soluble Peptide
Material in the RNA Fraction

	$\frac{E_{232}}{E_{260}}$	<u>Contribution of peptide material</u> <u>to absorption of RNA fraction</u>	
		at 260 m μ	at 232 m μ
Purified liver RNA	0.314	-	-
Acid-soluble "RNA" fraction (liver)	0.341	3%	7%
Purified thyroid RNA	0.500	-	-
Acid-soluble "RNA" fraction (thyroid)	0.609	11%	31%

to allow accurate corrections to be made. The choice of 232m μ as the second wavelength was made because it is the wavelength of minimal absorption of RNA and also at this wavelength polypeptides have a strong absorption due to the peptide bond peak at 200m μ (Tombs and MacLagan, 1962).

As previously described (Fleck and Munro, 1962), the RNA concentration of a solution can be computed from the formula: $C_{RNA} = A_{E_1} - B_{E_2}$ where A and B are constants, E_1 is the absorption of the mixture at one wavelength (λ_1), and E_2 is the absorption at (λ_2). The constants A and B are obtained from: $A = \frac{1}{r_1 - r_2 \cdot \frac{p_1}{p_2}}$ and $B = \frac{1}{r_1 \cdot \frac{p_2}{p_1} - r_2}$ r_1 and r_2 being the specific extinction coefficients of RNA at λ_1 and λ_2 , and p_1 and p_2 those of the acid-soluble polypeptide material at λ_1 and λ_2 .

Selecting the two wavelengths 260m μ and 232m μ , and using the $E_M(P)$ at 260m μ for calf-thyroid RNA (see Table 3), the concentration of RNA in terms of μ g. RNA-phosphorus/ml. solution in the acid-soluble fraction can be obtained from

$C_{RNA-P} = 3.79E_{260m\mu} - 1.50E_{232m\mu}$ - Equation (1). In a similar fashion, the concentration of RNA in terms of RNA-P

per ml. solution in an acid-soluble RNA fraction from rat liver can be obtained from $C_{\text{RNA}} = 3.40E_{260\text{m}\mu} - 1.44E_{232\text{m}\mu}$ - Equation (2) (Fleck and Begg, 1965). This method of correcting for the presence of polypeptide in acid-soluble "RNA" fractions from calf thyroid is illustrated in Table 1, and for rat liver in Table 2. Data for Table 2 is taken from studies of Fleck and Begg (1965). Tables 1 and 2 show that there is good agreement between the results obtained when the correction equation is applied and those based on direct estimation of polypeptide material present in the "RNA" fraction. Thus it can be seen that, although it is unnecessary to correct for the polypeptide material absorbing at 260m μ in the acid-soluble fractions from rat liver, corrections must be made in the case of the acid-soluble fractions from calf thyroid, since the error in assuming that the absorption at 260m μ is due to RNA alone would be about 12%, as stated previously.

Investigations into Methods of Estimating Thyroid DNA

Estimations of Deoxyribose

DNA fractions in 0.1N-NaOH from sheep, pig and calf-thyroid tissues were prepared without lipid extraction as described in the "Methods" section. Estimations of

deoxyribose in these fractions were then carried out according to Ceriotti (1952), and the absorption spectra of the colour complexes formed plotted over the range 440-520m μ and compared with that from a sample of purified calf-thymus DNA (Fig. 9). It can be seen from Fig. 9 that the absorption spectrum of the DNA fraction from calf thyroid is not identical with that of the calf-thymus DNA standard, although both have absorption maxima at 490m μ which is characteristic of deoxyribose. The thyroid sample appears to contain some interfering factor with an absorption maximum about 460m μ which results in a low $E_{490m\mu} / E_{460m\mu}$ ratio. Similar results were obtained when samples of sheep and pig-thyroid DNA fractions were used (Table 5). Since the values of the thyroid samples are higher than the equivalent standard at both ends of the spectrum (Fig. 9), the interfering factor must contribute to the absorption at 490m μ , and thus measurements of absorption at 490m μ will not provide accurate estimates of thyroid DNA. Alterations in the length of time and in the temperature of colour development did not alter the abnormal absorption spectra obtained from thyroid samples, and further extractions with chloroform did not remove the

Fig. 9.

ABSORPTION SPECTRA OF THE CERIOTTI DEOXYRIBOSE - INDOLE COMPLEXES
OF VARIOUS CALF-THYROID DNA FRACTIONS

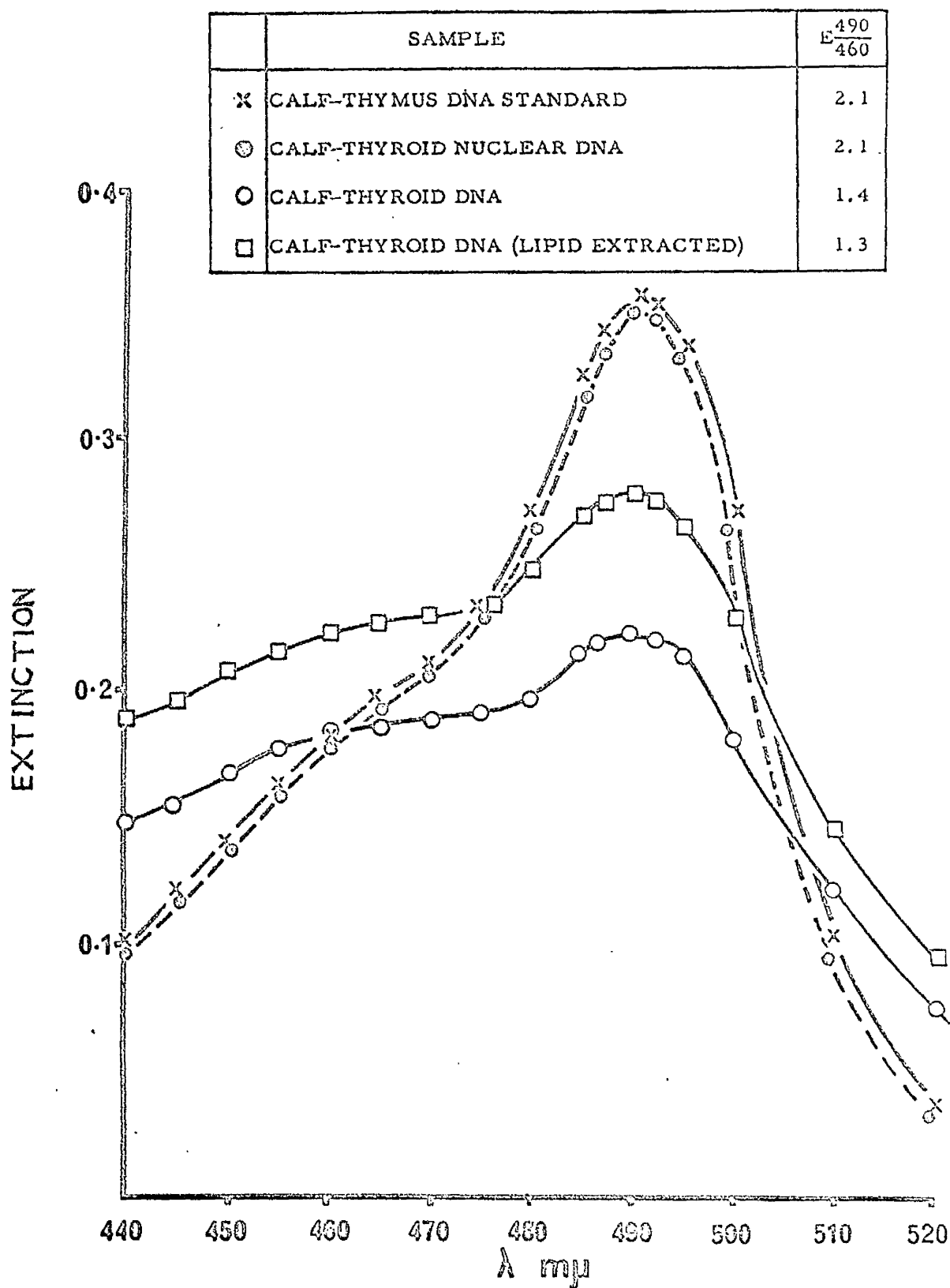


Table 5

Comparison of Estimates of DNA Content by Measurement of
Deoxyribose and DNA-Phosphorus in DNA Extracts
from Different Animal Thyroids

<u>Tissue DNA</u> <u>Extract</u>	<u>Number</u> <u>of Samples</u>	<u>Lipid</u> <u>Extraction</u>	<u>Ceretti Deoxyribose</u> <u>Estimations</u>	<u>Allen Phosphorus</u> <u>Estimations</u>
<u>Purified Calf -</u>				
Thymus DNA	6	-	2.10	2.46
Sheep Thyroid	4	-	1.79	2.36
Sheep Thyroid	4	+	1.88	2.92
Pig Thyroid	4	-	1.55	2.94
Pig Thyroid	4	+	1.61	3.04
Calf Thyroid	6	-	1.55	1.53
Calf Thyroid	6	+	1.57	1.67
			$\frac{E}{E}$ 490 460	(μ g. DNA-P) (μ g. DNA-P)
				2.43
				-
				2.79
				-
				2.90
				-
				1.51

interfering factor.

Thyroid DNA fractions were then prepared in duplicate from various thyroid tissues, one sample being prepared as described above, and the other lipid-extracted as described under "Methods". Samples were then taken for Ceriotti deoxyribose measurements and also for estimation of phosphorus (Allen, 1940) in the lipid-extracted samples. The results for samples of sheep, pig and calf-thyroid tissues are shown in Fig. 9 and Table 5. In all cases, the colour complexes produced in the Ceriotti reaction showed abnormal absorption spectra with low $E_{490m\mu} / E_{460m\mu}$ values, again showing the presence of some interfering factor in the thyroid DNA fractions. Also, in all cases, the samples which had undergone lipid extraction showed higher values for absorption at 490m μ and greater $E_{490m\mu} / E_{460m\mu}$ ratios than for the corresponding samples prepared without lipid extraction. The reason why lipid extraction causes an increase in absorption at 490m μ of the thyroid DNA fractions is unknown, but a similar effect has been observed with DNA fractions prepared from liver and adrenal tissue. Perhaps lipid extraction removes some tissue component which causes partial inhibition of formation

of the deoxyribose-indole complex.

When the DNA phosphorus values were compared with those calculated from deoxyribose measurement (Table 5), the values obtained from estimates of phosphorus were consistently slightly lower than those obtained by the Ceriotti procedure especially when lipids had been extracted. This difference probably gives a measure of the increment in absorption at 490m μ due to the interfering substance present in the thyroid DNA fractions.

Since the Ceriotti procedure proved unsatisfactory for determinations of thyroid DNA, investigations into other methods of DNA estimation were carried out. The Burton modification (Burton, 1956) of the diphenylamine colorimetric reaction was tried, but was also found to be unsatisfactory for thyroid tissue extracts since poor duplication of results was obtained. This is probably due to the presence of glycosidic peptides of thyroglobulin present in the DNA fraction, since glycoprotein and sialic acid are known to interfere in the diphenylamine reaction (Croft and Lubran, 1965).

From the review of Hutchison and Munro (1961), it would appear that the two colorimetric methods mentioned

above are the only ones which are normally satisfactory for the quantitative estimation of tissue DNA. The method of Ceriotti seems to be the more sensitive of the two, and is less subject to interference from other tissue constituents. It was therefore decided to try and modify this method to find a suitable means of measuring thyroid DNA.

Determinations of thyroid DNA were then carried out on samples of calf-thyroid DNA fractions prepared with and without lipid extraction using a modification of the Ceriotti procedure (Schmid, Schmid and Brodie, 1963). Schmid et al., (1963) studied the kinetics of the Ceriotti reaction, and determined the optimum conditions for the colorimetric estimation of DNA, including the influence of pH, temperature and time on the rates of DNA hydrolysis and colour production. Absorption spectra of the colour complexes obtained from calf-thyroid fractions were plotted over the range 440-520m μ , and compared with that of purified calf-thymus DNA treated in the same manner (Fig. 10). The absorption spectrum of the standard calf-thymus DNA was identical with that obtained using the original Ceriotti procedure, but those of the thyroid

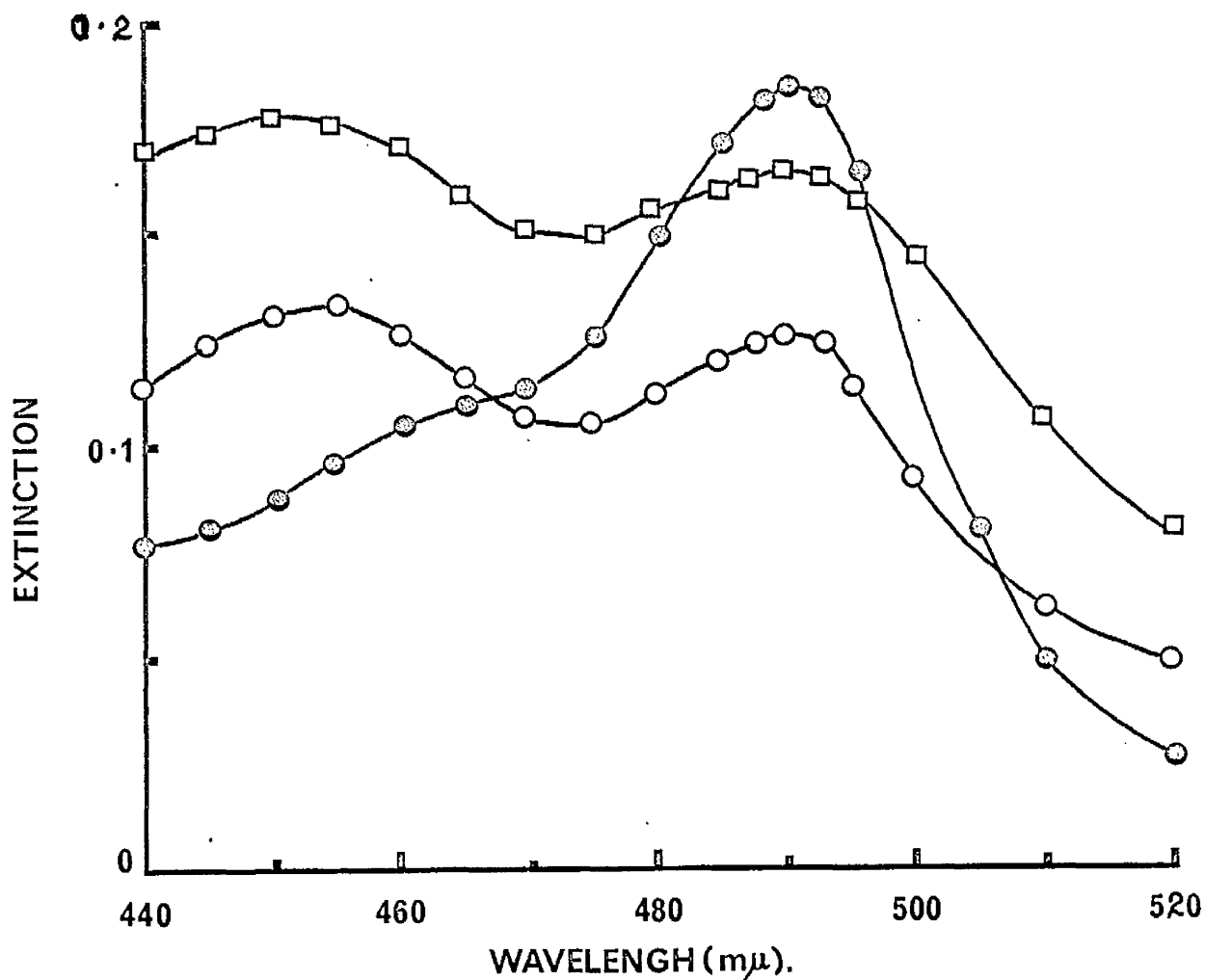


Fig. 10

Absorption spectra of deoxyribose-indole complexes of various DNA samples obtained using the method of Schmid et al. (1963). ●, calf-thymus DNA standard; ○, calf-thyroid DNA fraction; □, calf-thyroid DNA fraction (lipid extracted).

samples were again abnormal due to the presence of some interfering substance with an absorption maximum about 455m μ . Also the absorption at 490m μ was again greater for the lipid-extracted samples than for those which had not undergone lipid extraction. Thus this modification of the Ceriotti procedure is still unsatisfactory for the measurement of deoxyribose due to interference from some component of the thyroid DNA fractions.

Nature of the Factor interfering in the Ceriotti Deoxyribose Estimations

The other main constituent of the DNA fractions from thyroid tissue is acid-precipitable protein, in this case mainly thyroglobulin. Since thyroglobulin is an iodoprotein with a variety of carbohydrate residues attached to it, such as mannose, fucose, galactose, hexosamine, and sialic acid (Spiro and Spiro, 1965b), it could perhaps be the source of interference during colorimetric estimation of deoxyribose. It was found also that, the larger the mammal from which the DNA fraction was prepared, the greater the interference during deoxyribose determinations (see Table 5). This is in

agreement with the fact that, with increasing body size, the protein content of the thyroid gland increases, whereas the DNA content diminishes (see Table 6), and again suggests that protein (thyroglobulin) is the source of interference.

A sample of thyroglobulin (4mg), prepared by the method of Derrien et al. (1948), was subjected to the procedure of Fleck and Munro (1962) for the preparation of the DNA fraction, and Ceriotti estimations then carried out on samples of this fraction. The absorption spectrum of the colour produced by the thyroglobulin extract was plotted over the range 440-520m μ (Fig. 11). Fig. 11 shows that thyroglobulin gives a colour in the Ceriotti reaction which is not chloroform-extractable, and which absorbs at 490m μ . Therefore thyroglobulin is probably the source of interference during the colorimetric estimation of thyroid deoxyribose content.

Further evidence for this was obtained when the nucleic acids of purified nuclei from sheep-thyroid tissue (see Section II) were separated, and Ceriotti determinations carried out on the

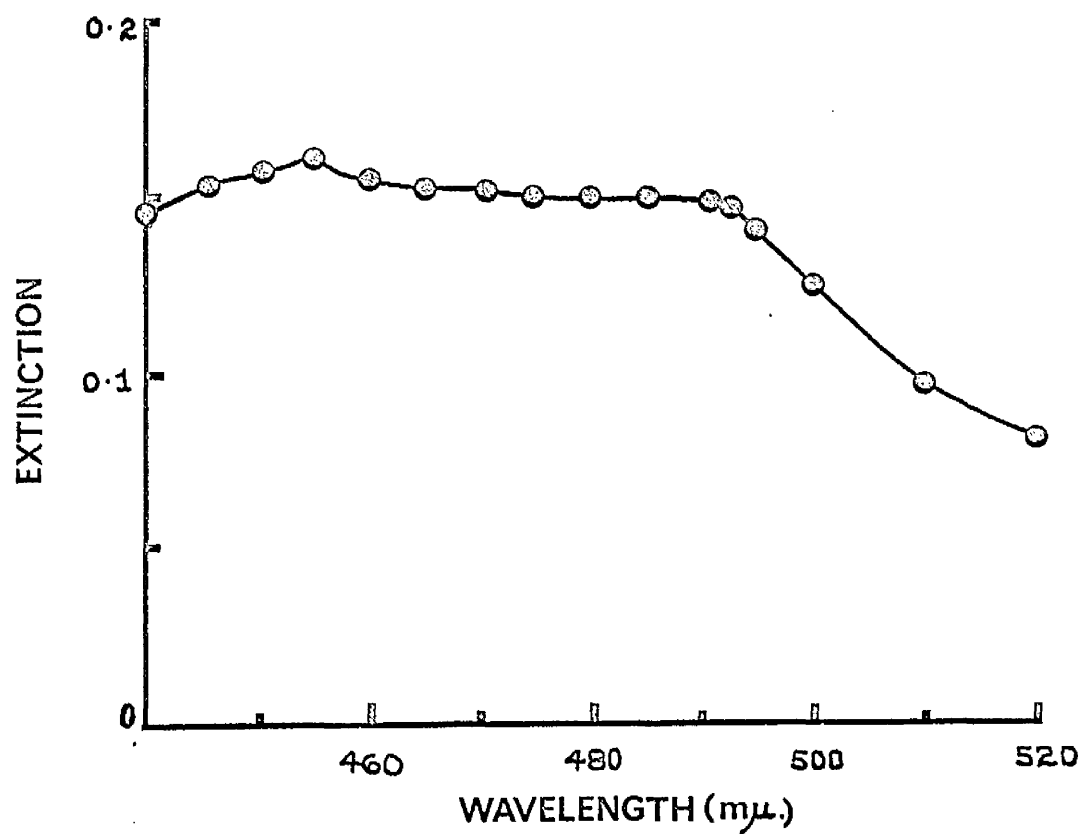


Fig. 11

Absorption spectrum of the colour complex produced by the acid-precipitable fraction of thyroglobulin (2mg. protein) in 0.1N-NaOH when reacted according to the procedure of Ceriotti (1952).

nuclear DNA fractions. In this case, where there is no thyroglobulin protein material present, the deoxyribose-indole complex obtained is identical with that from purified calf-thymus DNA (see Fig. 9). However it is still not known whether the interference during deoxyribose estimations is caused by the iodine or carbohydrate residues of thyroglobulin. When thyroxine (1mg.) was subjected to the Ceriotti procedure, it did not give rise to any colour complex absorbing in the range 440-520m μ , suggesting that the iodine residues of thyroxine at this concentration do not interfere in the Ceriotti reaction.

Several attempts were made to explore the interference due to thyroglobulin. According to Ceriotti (1952), estimations of deoxyribose can be carried out on samples of whole tissue homogenate without preparation of a DNA extract. Duplicate 2ml. samples of a 1 in 20 homogenate of calf-thyroid tissue were treated under Ceriotti conditions, except that, in one of the duplicates, 1ml. water replaced the indole. It was found that the samples not reacted with indole gave a yellow colour which was not chloroform-extractable and which absorbed at

490m μ , but with no absorption peak in the range 440-520m μ . The tissue samples given the full Ceriotti reaction with indole showed abnormal absorption spectra similar to that shown in Fig. 9. Thus on boiling with conc. HCl alone, some substance in thyroid tissue reacts and produces a yellow colour absorbing at 490m μ . This might be possibly due to liberation of iodine from thyroglobulin under the acidic conditions used. However thyroxine, on boiling with conc. HCl under the conditions of the Ceriotti reaction, did not appear to give rise to a colour complex which absorbed at 490m μ .

Duplicate samples of the DNA extracts of calf thyroid prepared with and without lipid extraction were then subjected to the Ceriotti procedure, but with one of the duplicates containing water in place of indole. The absorption spectra of the colour complexes produced were again plotted over the range 440-520m μ as shown in Fig. 12. It can be seen that the samples heated in the absence of indole contained material absorbing at 490m μ but with no absorption peak in the range plotted. The extinction at 490m μ was also greater for the sample which had undergone

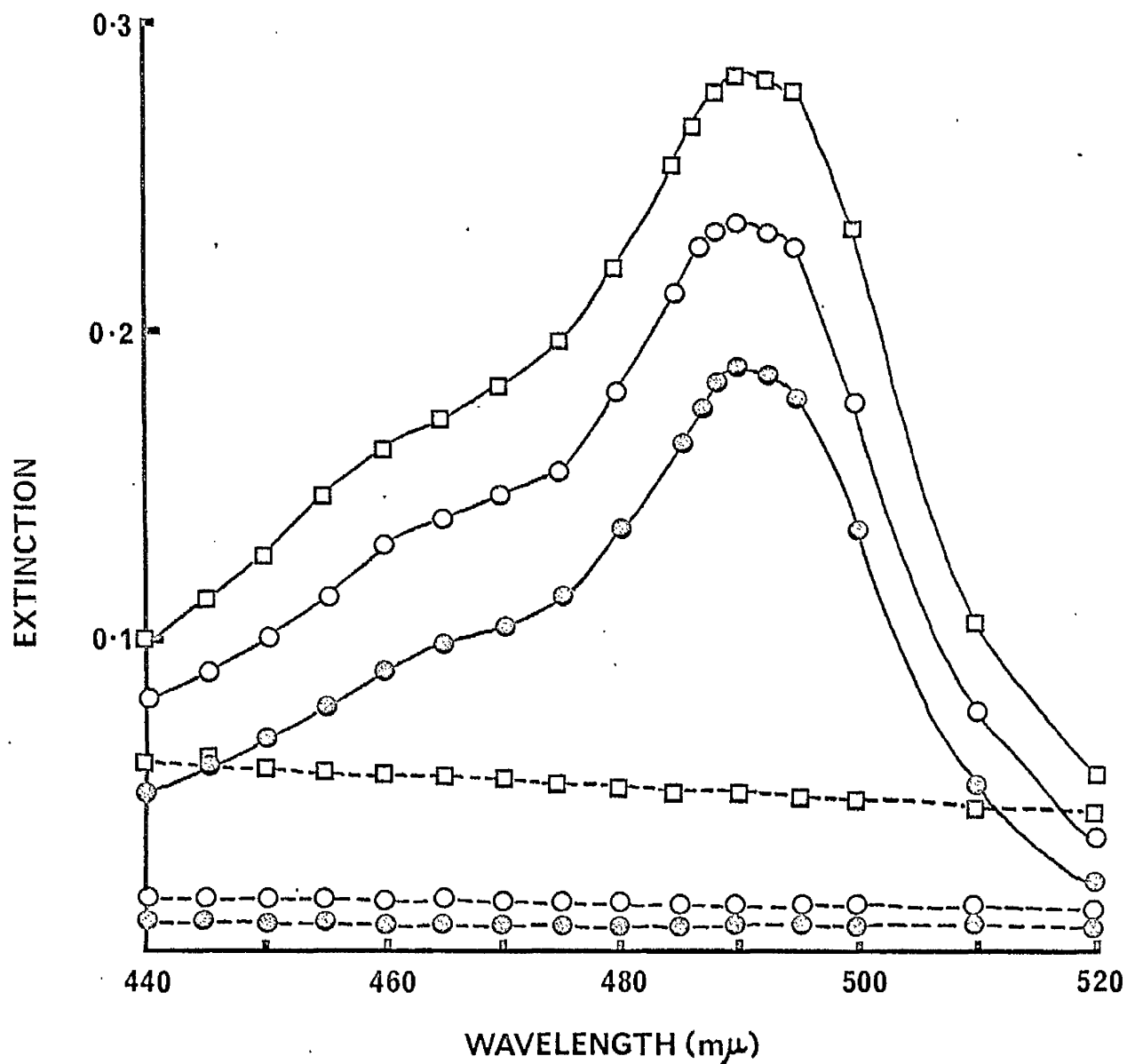


Fig. 12

Absorption spectra of various DNA samples treated according to Ceriotti (1952) with or without indole. The solid lines represent the samples treated in the presence of indole and the broken lines those treated in the absence of indole. ●, calf-thymus DNA standard; ○, calf-thyroid DNA fraction; ■, calf-thyroid DNA fraction (lipid-extracted).

lipid extraction. It was then considered that it might be possible to correct for the interference at 490m μ , presumably due to thyroglobulin, by subtracting the value of the absorption at 490m μ due to the samples heated in the absence of indole from those heated with indole, in order to obtain accurate estimates of deoxyribose. However on subsequent investigation this was found to be unsatisfactory, since the absorption at 490m μ of these control samples increased slowly on exposure to air, probably due to oxidation, and it was difficult to treat all samples under identical conditions.

Thus colorimetric estimations of deoxyribose on thyroid DNA fractions from whole tissue are generally unsatisfactory due to the presence of acid-precipitable residues of thyroglobulin occurring in the DNA fractions. Whether the interference is due to the iodine or carbohydrate residues of thyroglobulin, or both, is still not known.

Determination of DNA by Phosphorus Estimation

Since estimations of deoxyribose were subject to interference, it was decided to estimate thyroid DNA phosphorus by measuring the amount of phosphorus

occurring in the DNA fractions following lipid extraction, a procedure which has been found to provide satisfactory estimates of DNA content in other tissues (Hutchison and Munro, 1961). This method gave measurements of thyroid DNA content which were in the same range as the Ceriotti values but about 5-10% lower due to the enhanced values obtained by the colorimetric reaction. This suggests that, in the DNA fractions of the thyroid gland, there can be little phosphoprotein remaining in the DNA fraction, otherwise it would not be possible to equate the DNA-phosphorus estimates with those obtained by deoxyribose assay.

Comment on the Precision of Nucleic Acid Estimations

There are several requirements for the accurate estimation of RNA by the Schmidt-Thannhauser procedure. First, during cold acid precipitation of the tissue constituents, the optimum concentration of HClO_4 for this preliminary stage has been shown by Hallinan et al. (1963) to be 0.2N . In addition this concentration of HClO_4 does not hydrolyse RNA at 0° (Ogur and Rosen, 1950). Many authors follow this with extraction of lipids. That lipid extraction was a hazard was

first demonstrated by Venkataraman (1960), and was later confirmed when it was shown that ethanol or acetone applied after acid precipitation led to considerable losses of RNA (Hallinan et al., 1963).

However, the critical stage in the Schmidt-Thannhauser procedure is that of alkaline hydrolysis. Prolonged alkaline digestion in 0.3N or 1.0N-KOH or NaOH results in extensive extraction of non-RNA material in particular polypeptide which could for example account for 30-70% of the ultraviolet absorption of the acid-soluble fraction (De Deken-Grenson and De Deken, 1959; Fleck and Munro, 1962). Scott, Fraccastoro and Taft (1956) demonstrated that hydrolysis with 1.0N alkali for 1 hr. was sufficient for complete extraction of tissue RNA. This has been confirmed for rat-liver tissue (Fleck and Munro, 1962) and for several other tissues and bacteria, and it appears that hydrolysis for 1 hr. at 37° in 0.3N-alkali is usually satisfactory. From the studies carried out on thyroid tissue, it was found that a 1 hr. digestion at 37° in 0.3N-KOH was also sufficient for complete extraction of thyroid RNA.

However the hyperchromic effect appeared to be only about 96% complete at this time, in contrast to rat liver where the effect was fully developed after 1 hr. alkaline digestion. Also, even when the short digestion period was used, there was still considerable contamination of the thyroid acid-soluble "RNA" fractions with polypeptide material unlike rat liver where the contamination due to polypeptide was negligible. The contaminating polypeptides are alkaline digestion products of thyroglobulin which are acid-soluble. Thus corrections for this source of error have to be made before accurate estimates of thyroid RNA content by ultraviolet absorption measurements can be obtained.

Corrections may be made by determination of the amount of interfering peptide material in the "RNA" fractions by the method of Lowry et al. (1951) providing a pure sample of polypeptide is available for direct comparison. Although the method is straightforward, the possibility of employing a more rapid procedure was considered. The presence of significant amounts of polypeptide in acid-soluble "RNA" fractions can also be detected by comparing the $E_{280m\mu} / E_{260m\mu}$ and $E_{232m\mu} / E_{260m\mu}$

ratios of this fraction with those of purified tissue RNA. When the shorter period of alkaline digestion is employed, interference due to protein is not great, so that the contribution of polypeptide to the absorption at 270-280m μ is not usually large. The selection of 232m μ as the second wavelength gives a considerable increase in sensitivity in the detection of peptide so that accurate corrections may be made. It is essential to use as standard the appropriate tissue RNA because the absorption spectra of the RNA of various tissues from different species are different (Magasanik, 1955; Fleck and Munro, 1962).

The requirements for the reliable estimation of RNA by the Schmidt-Thannhauser procedure thus are: in the preliminary stages, the use of 0.2N-HClO₄ as precipitant and the omission of lipid solvents; the use of 1 hr. digestion at 37° with 0.3N-KOH; availability of a pure sample of the tissue RNA and the determination of whether interference due to polypeptide is significant. To correct for polypeptide, a representative sample of the interfering material should be prepared and from this a rapid and accurate two-wavelength

ultraviolet absorption procedure can be developed.

Measurements of thyroid DNA were carried out on the DNA fractions prepared by the modified Schmidt-Thannhauser procedure by estimation of deoxyribose or phosphorus provided removal of phospholipids had been carried out. From the review of Hutchison and Munro (1961) it would appear that the two procedures which are most satisfactory for estimation of deoxyribose are the colorimetric methods of Ceriotti (1952) and of Burton (1956). However both these methods are subject to interference from other tissue components occurring in the DNA fractions.

It was found from studies of the absorption spectra of the colour complexes produced in the Ceriotti reaction that the spectra of the thyroid tissue samples were not identical with that of a sample of purified DNA due to the presence of some interfering factor present in the thyroid DNA fractions. Thus measurements of DNA by deoxyribose estimation are inaccurate due to this source of interference. Attempts to remove the contaminating material or alter the method of deoxyribose assay were not successful, and an alternative

means of estimating thyroid DNA was employed.

Estimates of the phosphorus content of the lipid-extracted DNA fractions were then carried out (Allen, 1940). This method has proved satisfactory in several other tissues (Hutchison and Munro, 1961), but errors could arise if the tissue under investigation contained significant amounts of phosphoprotein the phosphorus of which is not acid- or lipid-soluble thereby increasing the phosphorus content of the DNA fraction. However, for thyroid tissue, the values obtained by phosphorus measurement were in the same range as those obtained by measurement of deoxyribose, and thus errors arising from this source are not significant. Therefore, when measurements of tissue DNA are being carried out, comparisons of the results obtained using different procedures, e.g. by estimations of deoxyribose and phosphorus, should be made to ensure that other tissue components present in the DNA fractions are not contributing to the values obtained during the assays.

Thyroid Composition and Body Size

The methods developed above were then applied to analysis of the thyroid glands of various mammals. Table 6 displays the analytical data obtained on the thyroid glands of mammals ranging in weight from 200g.(rats) to 400kg. (cows). With increasing body size, the composition of the gland undergoes progressive changes. The percentage of protein in the tissue increases considerably with increase in body size, whereas the proportion of the other constituents diminishes, especially that of RNA.

In order to obtain equations describing the effect of body size on thyroid composition, the logarithms of the amounts of each thyroid constituent were plotted against the logarithms of body weight (Fig. 13); the data for man were not used for this purpose since only one human gland had been analysed. In the case of each thyroid constituent, the data after logarithmic transformation can be fitted to a straight line relationship and are thus susceptible to description by a regression equation such as Brody (1945) has used for relating organ size to body weight. The equation takes the form

Table 6

Protein, Nucleic Acid and Phospholipid Content
of Thyroid Glands of Mammals arranged
in Order of Body Weight

Species (in order of size)	Number analysed	Avg. Body wt. (Kg.)	Composition/100g. wet wt. thyroid*			
			Protein (g.)	RNA (mg.)	DNA (mg.)	Phospholipid (mg.)
Rat	32	0.2	9.1 ± 0.3	259 ± 13	229 ± 6	606 ± 30
Rabbit	4	2.2	11.9 ± 0.6	249 ± 4	238 ± 6	587 ± 19
Cat	4	3.0	12.4 ± 0.8	245 ± 12	252 ± 14	509 ± 16
Dog	4	16.5	14.0 ± 0.7	214 ± 11	244 ± 15	493 ± 13
Lamb	6	20.2	20.5 ± 0.7	182 ± 5	276 ± 7	496 ± 25
Man	1	70	18.7	128	193	429
Pig	4	148	19.9 ± 0.6	123 ± 24	192 ± 11	472 ± 14
Cow	5	418	23.5 ± 1.3	138 ± 20	180 ± 10	470 ± 27

*Mean values with standard errors

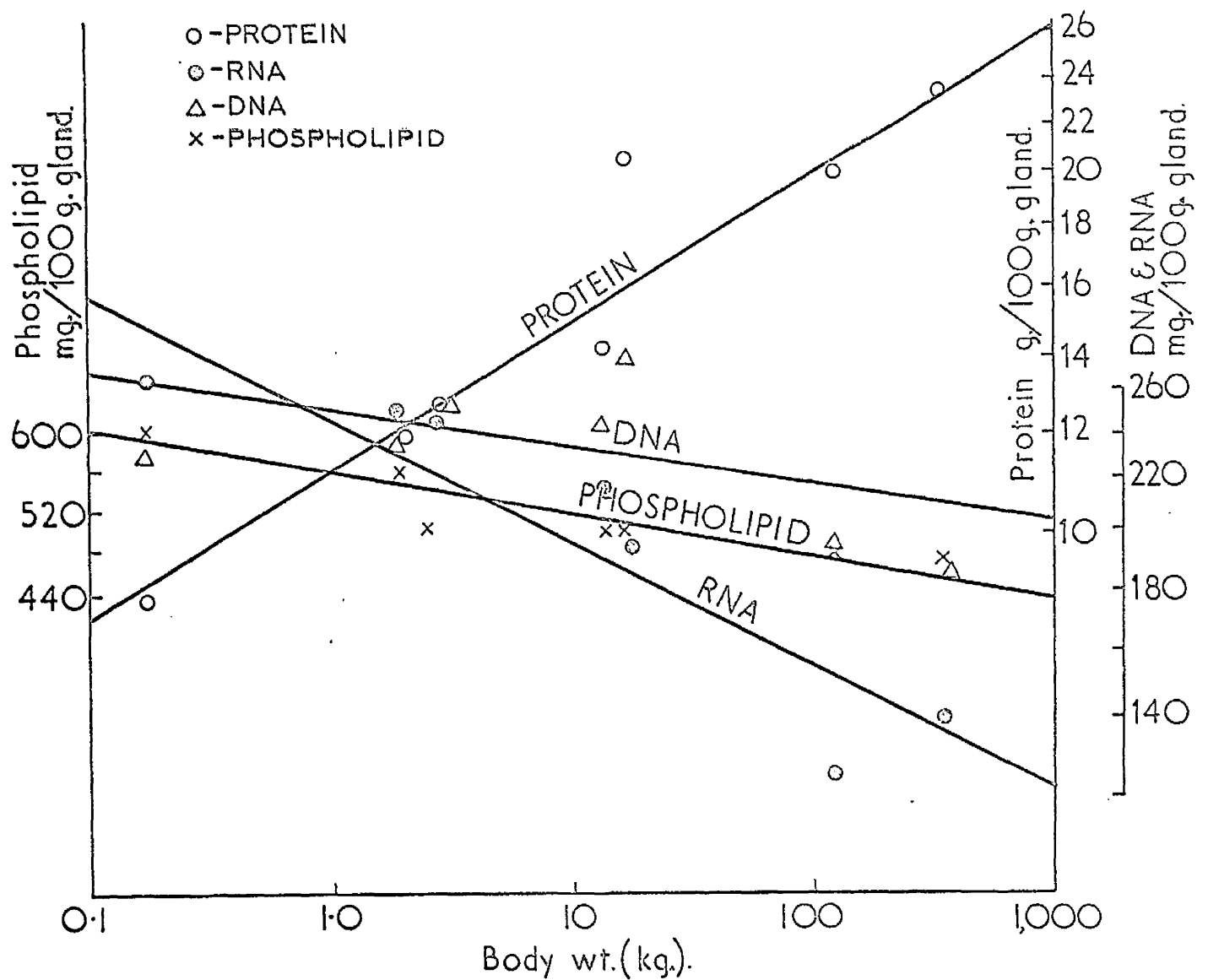


Fig. 13

Regression lines showing the concentrations of thyroid protein, DNA, RNA and phospholipid in relation to the body weight of different mammals.

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$\log S = \log a + x \log W$, where S is the substance measured, a is a constant, W is the body weight, and x is the slope of the line relating $\log S$ to $\log W$. When transcribed into the arithmetic form, the equation becomes $S = aW^x$. The equations of the slopes of the lines, with standard errors of the estimates of the mean values, relating the thyroid constituents to body weight in kg are shown in Table 7 (column 1). Protein is expressed in terms of g/100g thyroid gland, whereas RNA, DNA and phospholipid are expressed as mg/100g thyroid gland. Following statistical analysis of the data obtained from individual animals (Table 6) by analysis of variance in a two-way classification, the results in Fig. 13 and Table 7 show that body size has a significant effect on protein concentration, RNA concentration and phospholipid concentration ($P < 0.01$), but not on DNA concentration. The equations in Table 7 confirm that protein concentration in the gland increases with body size as shown by the positive exponent of body weight (+0.123), whereas the other constituents diminish and thus have negative exponents (-0.104, -0.033, and -0.036).

Table 7Regression Equations for Relationship of
Thyroid Constituent to Body Wt.

<u>Constituent</u>	<u>% Composition</u>	<u>10² x Total Amount per Gland*</u>
Protein (g.)	11.0 \bar{W} + 0.123 \pm 0.019	1.42 \bar{W} 1.051
RNA (mg.)	251 \bar{W} - 0.104 \pm 0.021	32.4 \bar{W} 0.824
DNA (mg.)	248 \bar{W} - 0.033 \pm 0.021	32.0 \bar{W} 0.895
Phospholipid (mg.)	564 \bar{W} - 0.036 \pm 0.007	72.7 \bar{W} 0.892

*Derived using Brody equation for relationship between body wt. (\bar{W} in Kg.) and thyroid mass (\bar{T} in g.) $\bar{T} = 0.129\bar{W}^{0.928}$

From examination of an extensive range of mammals, Brody (1945) found that the weight of the thyroid gland varies as the 0.928 power of body weight. Data for the relationship of thyroid weight to body size of the animals used in the analytical experiments are shown in Table 8. From our results thyroid weight was found to vary as the 1.09 power of body weight. However this relationship is based on fewer animals and a smaller range of species than Brody examined, and indeed the value (1.09) may not be significantly different from the value of Brody's exponent (0.928). In consequence, the value (0.928) will be accepted as a more accurate evaluation of the relationship of thyroid size to body weight.

Using Brody's equation relating thyroid weight to body size, we can compute the effect of body weight on the total amount of each constituent of the gland by combining the exponent 0.928 with the relationships shown in Fig. 13 and Table 7 (column 1) for the individual chemical components of the gland. The derived equations for the total amounts of thyroid constituents per kg. body weight are shown in Table 7 (column 2) and are also expressed graphically (Fig. 14).

Table 8

Weights of Thyroid Glands of Different
Mammals Arranged in Order of Body Size

<u>Species</u> (in order of size)	<u>Number Analysed</u>	<u>Avg. Body Wt.</u> (kg.)	<u>Avg. Thyroid Wt.</u> (g.)
Rat	32	0.2	0.012
Rabbit	4	2.2	0.217
Cat	4	3.0	0.319
Dog	4	16.5	1.33
Lamb	6	20.2	3.28
Pig	4	148	24.6
Cow	5	418	44.35

Equation for relationship between Body Wt.
(W in kg.) and Thyroid Wt. (T in g.) :-
$$T = 0.104W + 1.090 \pm 0.047$$

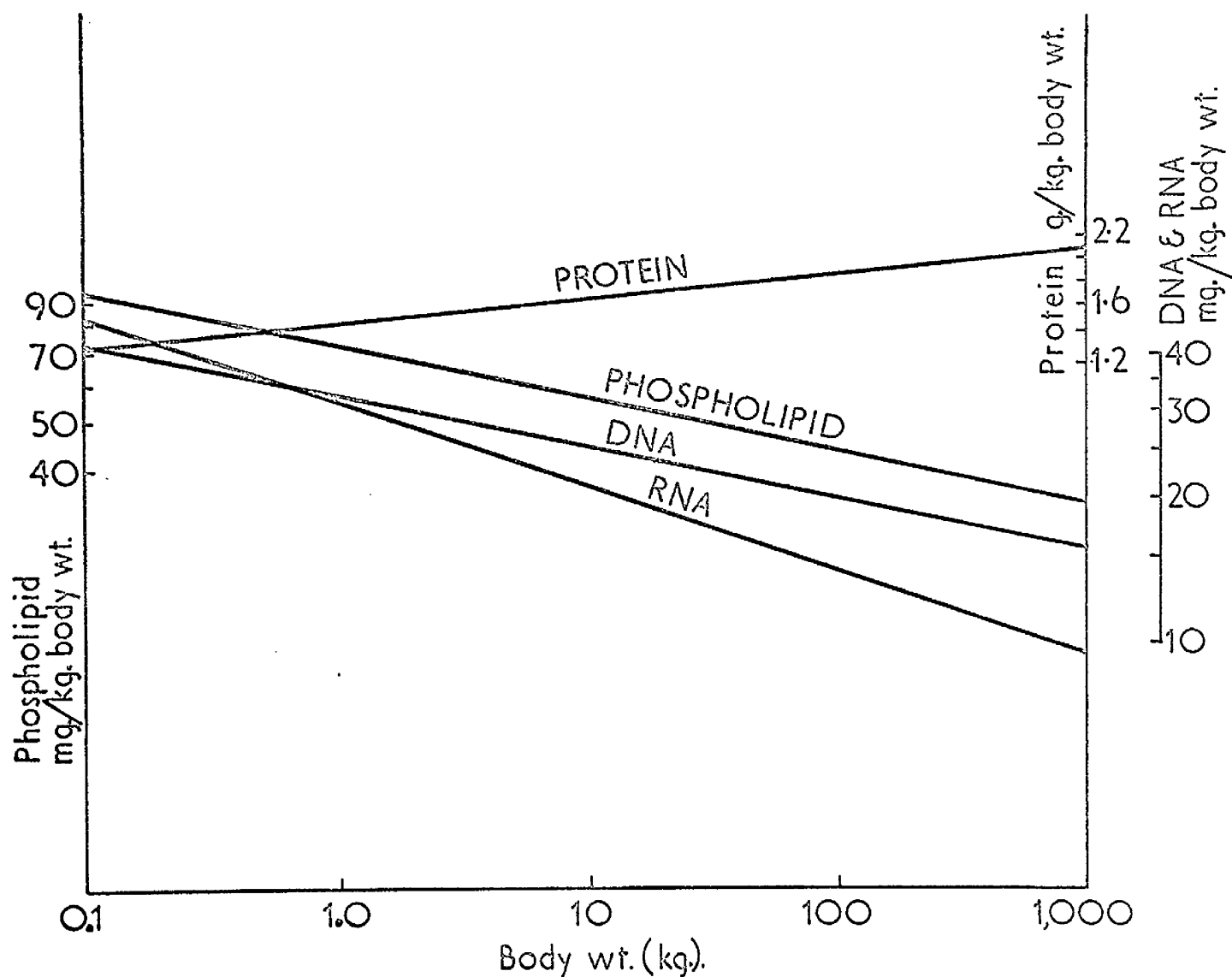


Fig. 14

Regression lines showing the total quantities/kg. body weight for protein, DNA, RNA and phospholipid in the thyroid glands of different mammals.

Note:- Ordinates show the values $\times 10^2$ for the total quantities of protein (g.) and DNA, RNA and phospholipid (mg.).

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These calculations show that the relatively small size of the thyroid gland in large mammals ($W^{0.928}$) is offset by the increase of protein ($W^{0.123}$) so that, over the range of mammals studied, the total amount of protein in the thyroid gland parallels body weight ($W^{1.05}$). Consequently the amount of thyroid protein per kg. body weight does not diminish with increasing body size (Fig. 14). On the other hand, this picture does not apply to the total amounts of the other thyroid constituents which are proportionately reduced in the larger species (Fig. 14), and are therefore related to body weight by exponents less than unity ($W^{0.824}$, $W^{0.895}$, and $W^{0.892}$).

In the case of DNA, this means that larger mammals have fewer thyroid cells relative to their body weight, since all mammals have the same amount of DNA per cell, except for the occurrence of polyploidy in such cases as rat and mouse liver (Vendrely, 1955). Consequently we can use DNA as a measure of cell number in order to express the amounts of the other thyroid constituents on a cell basis. Table 9 shows the amounts of the various thyroid constituents per unit

Table 9

Amounts of Thyroid Constituents per unit
of DNA and the Dimensions of Thyroid
Follicles in Different Mammals

Species (in order of size)	<u>Amounts/ mg. DNA*</u>			<u>Follicle Size†</u>	
	<u>Protein</u> (mg.)	<u>RNA</u> (mg.)	<u>Phospholipid</u> (mg.)	<u>Long diam.</u> (μ)	<u>Short diam.</u> (μ)
Rat	39.8	1.13	2.65	117	77
Rabbit	50.2	1.05	2.43	131	101
Cat	49.1	0.97	2.02	156	127
Dog	57.2	0.88	2.02	277	139
Lamb	74.3	0.66	1.80	-	-
Man	96.7	0.66	2.21	-	-
Pig	104.0	0.64	2.46	294	225
Cow	130.4	0.77	2.62	337	237

†Mean dimensions of 50 largest follicles; taken from histological studies of Yagizawa (1956).

*There is a significant regression of protein/ mg. DNA on body weight ($P < 0.01$), and of RNA/ mg. DNA on body weight ($P < 0.02$), but not for phospholipid/ mg. DNA.

of DNA and it can be seen, that, over the range of mammals studied, the amount of protein in the gland per thyroid cell increases $4\frac{1}{2}$ times, whereas the amount of RNA in the cell decreases to half. Statistical analysis of the data was carried out by analysis of variance, and it was shown that there was a significant regression of protein per mg. DNA on body weight ($P < 0.01$), and of RNA per mg. DNA on body weight ($P < 0.02$), but not of phospholipid per mg. DNA on body weight.

The significance of these findings must be related to the morphological appearance of the thyroid gland in various mammals. From histological studies made on a variety of mammals (Teissier, 1939), there is no evidence of any relationship of thyroid cell dimensions to body size, and thus it seems unlikely that the considerable increase in protein content in the larger mammals can be due to the presence of more protein in the cytoplasm of the thyroid cells. On the other hand, measurements of thyroid follicle dimensions in a range of mammals has demonstrated a consistent enlargement of the vesicles as species size increases (Yagizawa, 1956), and a similar relationship of follicle size to body weight has been

observed in birds (Yamamoto, 1959). Yagizawa's data are reproduced in Table 9, and show that his reported increase in mammalian follicle size in larger mammals correlates closely with the accumulation of protein in the gland. This leads to the conclusion that the extra protein per unit of DNA observed in the larger mammals is in fact stored in the enlarged follicles.

In presenting this general picture of significant changes in thyroid gland composition with body size, it is important to point out that individual species may not necessarily fit closely to the general pattern described above. In particular, published compilations (Brody, 1945; Spector, 1956a; Long, 1961) of thyroid weights in different species show that the human thyroid is exceptionally large in relation to body weight. Thyroid size will also be affected by diet, particularly by iodine uptake; we must assume that these effects are random in the mammalian series studied.

General Significance of the Changes in Thyroid Composition

Mammalian metabolic processes are affected to a considerable extent by the size of the animal. Both energy

metabolism (Brody, 1945) and protein metabolism (Munro, 1964) diminish in intensity with increasing body size, each being related to approximately the 0.7 power of body weight. It might therefore be expected that endocrine organs regulating metabolism would be much smaller in proportion to body weight in the larger mammals. As Brody (1945) has shown, this predicted relationship ($W^{0.7}$) of endocrine size to body weight is nearly fulfilled in the case of the pituitary gland ($W^{0.762}$) and of the adrenal gland ($W^{0.798}$). On the other hand, the mass of the thyroid gland relative to body weight was found by Brody (1945) to be much less influenced by body size, the relationship being to the 0.928 power of body weight.

The analysis of thyroid composition carried out shows that the individual chemical components of the gland do not share equally in this relationship of thyroid mass to body weight. The total amount of one major thyroid constituent, protein, remains a constant proportion ($W^{1.05}$) of body weight over the range of mammals, whereas the total quantities of the other thyroid constituents measured become less in proportion to body weight as the size of the mammal increases

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(Fig. 14). From histological evidence (Teissier, 1939; Yagisawa, 1956), it is apparent that this differential effect is achieved by a change in the ratio of cells to colloid, the larger mammals having bigger follicles with proportionately fewer cells in relation to colloid. Thus the larger mammals maintain a constant relationship between the amount of protein in the thyroid gland and body weight by more extensive storage of protein (thyroglobulin).

There is an analogy between this picture of a cell population that becomes proportionately smaller as body size increases while maintaining an extracellular pool of protein that is constant in relation to body size and the relationship, among different mammals, of the size of the liver to its extracellular secretion the plasma proteins. Over the range of mammals, liver mass increases less rapidly than body weight ($W^{0.87}$) whereas the volume of plasma per unit of body weight and its protein content are not systematically influenced by species size (Spector, 1956b). Irrespective of liver size, mammals maintain the same amount of plasma protein per unit of body weight, and the larger species achieve this by a

reduction in rate of turnover. Munro and Downie (1964) showed that this reduction in the intensity of plasma albumin synthesis and breakdown by larger mammals coincides with a smaller amount of RNA per unit of DNA in the liver cell, a not unexpected finding since RNA is concerned with protein synthesis. Like the liver, the thyroid glands of the larger mammals also contain less RNA per unit of DNA (Table 9), and it is thus likely that the turnover of thyroid protein is slower in larger than in smaller species of mammal. At present, however, there is no published data available on the rate of synthesis and degradation of protein in the thyroid glands of different species.

SUMMARY

1. Investigations have been carried out on the application of standard methods for the estimation of nucleic acids to thyroid tissue. The presence of thyroglobulin gave rise to certain errors not encountered in other mammalian tissues, and modified analytical procedures were therefore devised. In attempts to estimate RNA in samples of thyroid tissue by a modified Schmidt-Thannhauser method using ultraviolet absorption

measurements following a 1-hr. digestion in 0.3N-alkali, interference due to the presence of polypeptide material was encountered and a two-wavelength correction procedure was devised which eliminates errors from this source. Deoxyribose estimations were carried out, using various procedures, on thyroid DNA fractions prepared using the modified Schmidt-Thannhauser method, but were found to be unsatisfactory due to interference from alkaline hydrolysis products of thyroglobulin present in these DNA fractions. DNA was therefore measured by estimation of DNA phosphorus in lipid-extracted DNA fractions.

2. With increasing size of mammal, the concentration of protein in the thyroid gland increases, whereas there is a diminution in the concentration of RNA and, to a smaller extent, of DNA and phospholipid. These findings can be correlated with an increase in follicle size in larger mammals, which results in more colloid in proportion to the number of cells. Calculations made from the data show that the amount of protein in the thyroid glands of various mammals is maintained at a constant proportion of body weight. However, the number of cells in the thyroid gland does not increase in parallel with the

size of the species, and the larger mammals also have less RNA per cell. These observations suggest that the turnover of protein in the thyroid glands of larger mammals may be less rapid than in smaller mammals.

SECTION II

Studies of the Action of TSH on RNA and
Protein Metabolism in the Thyroid Gland

INTRODUCTION

Prolonged administration of TSH causes an increase in the RNA content of the thyroid gland followed by an increment in DNA content due to cell multiplication

(Matovinovic and Vickery, 1959; Ekholm and Pantić, 1963).

Using calf-thyroid slices, Hall (1963) demonstrated that TSH increased the uptake of (^{14}C) formate by the purine bases of tissue RNA and also increased the incorporation of (^{14}C) adenine into tissue RNA after incubation for 3 hr. and he attributed this effect to stimulation of synthesis of purine ribonucleotides caused by greater formation of ribose in the presence of TSH. However, no investigations had been carried out on this effect of TSH at times earlier than 3 hr. or on the nature of the subcellular components and the species of RNA involved in this process. Therefore experiments were carried out to examine this effect in greater detail, particularly the sequence of events at various time intervals during incubation.

Many studies on the actions of various hormones on the nucleic acid metabolism of their target organs have been carried

out using either in vivo or in vitro experiments on normal laboratory animals such as rats or rabbits. However, in the case of the thyroid gland, this is not feasible since the thyroid glands of these animals are too small for detailed investigations of this nature as shown by the data obtained from the species analysis in Table 6. Thus studies had to be carried out in vitro using thyroid tissue obtained from larger mammals, in this case sheep. This however leads to further difficulties. For example, animal variation is great since the animals are not maintained under standard conditions, particularly with respect to diet and temperature. The activity of the thyroid gland is affected by iodine intake and by the presence of goitrogens in foodstuffs and is also stimulated during cold weather. Another difficulty arose due to the unavoidable time-lag after killing and removal of the glands and before the tissue was fractionated for in vitro assays. In some cases this led to the failure of the experiments since, although the glands were chilled as quickly as possible after removal from the animals, a variable degree of autolytic activity, particularly degradation of RNA, was found to occur during this time interval.

These effects resulted in great variations in activity of different thyroid tissue preparations. The results obtained from these experiments on the effect of TSH on thyroid RNA metabolism using tissue slices and purified nuclei from sheep-thyroid glands are described in the following section.

MATERIALS AND METHODS

Chemicals used

(8-¹⁴C) adenine sulphate (31.3mc/m-mole) and (1-¹⁴C) DL-leucine (36.4mc/m-mole) were obtained from The Radiochemical Centre, Amersham, Bucks. ATP, CTP, GTP and UTP and crystalline bovine pancreatic DNase I were from the Sigma Chemical Co., St. Louis, Mo., U.S.A. TSH, "thytropar" was from the Armour Pharmaceutical Co., Illinois. Puromycin in the form of the dihydrochloride was from the Nutritional Biochem. Corp., Cleveland, Ohio, and actinomycin D was a gift from Messrs. Merck, Sharpe and Dohme Inc., Rahway, N.J. (³²P) UTP labelled in the α -phosphorus atom was a gift from Dr. R.M.S. Smellie, and (³²P) UTP labelled in the $\beta\gamma$ -phosphorus atoms was obtained from Schwarz Biochem. Inc., Orangeburg, N.Y.

Experiments on Tissue Slices

Preparation of Thyroid Slices and

Incubation Conditions

Thyroid glands were excised from sheep as soon as possible after killing at the local abattoir and transported to the

laboratory in ice. All extraneous tissue was then removed and slices of thyroid tissue prepared using a chilled McIlwain chopper (McIlwain and Buddle, 1953).

Sheep-thyroid slices (about 400mg) were suspended in 4ml. Krebs-Ringer phosphate buffer pH 7.4 (Umbreit, Burris and Stauffer, 1957) containing glucose (1mg/ml) and an amino acid mixture (Eagle, 1959) together with penicillin (100 units/ml) to eliminate bacterial growth during incubation. ($8\text{-}^{14}\text{C}$) adenine ($5\mu\text{c}$) was added to all samples and TSH to the test samples to give a final concentration of 0.1 i.u./ml. incubation medium. Incubations were then carried out at 37° with continuous shaking over a 3-hr. period in an atmosphere of oxygen. Samples from each batch were taken at zero time, as controls, and at various time intervals thereafter up to 3 hr. At the end of incubation the samples were quickly chilled in ice before centrifuging down the slices.

In one series of experiments the TSH and (^{14}C) adenine were added at the start of incubation as before and puromycin ($100\mu\text{g}/\text{ml}$) or actinomycin D ($10\mu\text{g}/\text{ml}$) added at various times (0, 1 or 2 hr.) during the course of the 3-hr. incubation. Duplicate

samples with or without TSH but not containing these inhibitors were also incubated for 3 hr. at 37° to act as controls.

RNA Estimations and Radioactivity Measurements on Whole Cell, Nuclear and Cytoplasmic Fractions

After cooling in ice, the tissue slices were centrifuged down at $100g$ for 10 min. at 0° and washed with fresh ice-cold Krebs-Ringer buffer to remove free isotope and TSH. The tissue slices were then homogenized in 10 volumes ice-cold water or in 10 volumes ice-cold $0.25M$ -sucrose containing $0.001M$ - $MgCl_2$ and $0.002M$ - $CaCl_2$ if tissue fractionation was to be carried out. For separation into nuclear and cytoplasmic fractions the homogenate was centrifuged at $100g$ for 5 min. to remove debris and unbroken cells. The supernatant fluid was layered on to an equal volume of cold $0.35M$ -sucrose containing $0.001M$ - $MgCl_2$ and $0.002M$ - $CaCl_2$ and a crude nuclear fraction spun down at $1,000g$ for 10min., the cell cytoplasm remaining in the lighter fraction. The nuclei were then purified by centrifuging through $2.2M$ -sucrose as described later in this section (see Fig. 15).

The RNA contained in the whole tissue, nuclear and

cytoplasmic fractions was separated as described in Section I by the modified Schmidt-Thannhauser procedure of Fleck and Munro (1962). The fractions were treated with ice-cold HClO_4 (containing adenine at 2mg/ml. to dilute out contaminating isotope) to give a final concentration of 0.2N with respect to acid. Bovine serum albumin (2mg) was added to the nuclear samples to ensure complete precipitation of nucleic acids. The samples were then washed 3 times with 5ml. cold 0.2N- HClO_4 to remove acid-soluble small molecules and the tissue residues of protein and nucleic acids digested for 1 hr. at 37° in 0.3N-KOH. The DNA and protein were then removed by acidifying the digests to 0.2N with HClO_4 followed by centrifugation, the RNA remaining in the supernatants. Aliquots of the RNA fractions were then removed for radioactivity measurements and treated in the following manner. The samples were neutralised with KOH and, after standing 10 min. in ice, the precipitate of KClO_4 was centrifuged down and the supernatants containing the RNA plated on to metal planchettes using lens paper discs to give uniform self-absorption and counted in a Nuclear Chicago gas-flow counter. The remainder of the acid-soluble RNA fractions

was made up to a suitable volume in 0.1N-HClO₄ for estimation of RNA by ultraviolet absorption measurements correcting for polypeptide interference in the whole cell and cytoplasmic fractions as described in Section 1. In the case of the nuclear fractions, however, RNA was determined directly from the ultraviolet absorption at 260mμ taking an extinction value of 1.000 to be equivalent to 32μg.RNA since there was negligible interference due to polypeptide in these RNA fractions. The specific activities of the RNA fractions were then calculated in terms of counts/min.mg.RNA.

Preparation of Bentonite

It has been reported that bentonite binds to and inhibits ribonucleases (Brownhill, Jones and Stacey, 1959), and therefore it was used during preparation of RNA from sheep-thyroid tissue.

Bentonite suspensions were prepared from commercial bentonite (British Drug Houses Ltd., Poole, England). Bentonite (2g) was suspended in 40ml. water and centrifuged at 750g for 15 min. the sediment was discarded and the supernatant material was centrifuged at 8,700g for 20 min. The sediment so obtained was resuspended in 0.1M-EDTA (pH7.0) and stored in this solution

for 48 hr. at room temperature. The material was centrifuged once more at 750g and at 8,700g. The 8,700g sediment was suspended in 0.01M-sodium acetate (pH 6.0), centrifuged at 8,700g and the sediment taken up in the acetate buffer at a concentration of about 5% (w/v).

Preparation of Purified RNA from Sheep-Thyroid Slices

Slices of sheep-thyroid tissue were incubated with or without TSH for 3 hr. at 37° and the slices washed with fresh ice-cold Krebs-Ringer buffer after incubation as described above. RNA was then prepared from the tissue slices using a method similar to that described by Eason, Cline and Smellie (1963). The slices from each sample were homogenized in 5ml. ice-cold 0.01M-sodium acetate buffer (pH 6.0) containing 1% sodium dodecyl sulphate and 1mg% bentonite. The homogenate was then shaken with an equal volume of 90% (w/v) phenol containing 0.1% 8-hydroxyquinoline for 15 min. at room temperature. The solution was then spun at 30,000g for 15 min., the aqueous layer removed and bentonite added to it to give a final concentration of 1mg%. The phenol layer was washed twice with 0.01M-sodium acetate buffer (pH 5.25) and the aqueous extracts combined. This solution

was then extracted 3 times with an equal volume of ether, made 2% with respect to potassium acetate, and the RNA precipitated with 2 volumes ethanol for 1 hr. at -10° . The RNA was collected by centrifugation at 2,000g for 15 min. at 0° and the precipitate dissolved in 3ml. 0.01M-tris-HCl buffer (pH 7.5) containing 0.001M-MgCl₂ and 1mg% bentonite. Pancreatic DNase (5µg) was added to the solution which was incubated at 37° for 10 min. to degrade the DNA present to deoxyoligonucleotides. The solution was cooled, the RNA precipitated by addition of 2 volumes ice-cold ethanol, and after collection by centrifugation was dissolved in about 0.5ml. 0.01M-sodium acetate buffer (pH 5.25) containing 0.05M-NaCl. This solution was then dialysed for 18 hours at 4° against 2 litres 0.01M-sodium acetate buffer (pH 5.25) containing 0.05M-NaCl. The RNA solution was then centrifuged at 1,000g for 10 min. to remove a precipitate of bentonite.

Separation of Purified RNA on Sucrose Density Gradients and Radioactivity Measurements

Ultraviolet absorption measurements were carried out on the RNA samples and about 300µg. RNA in 0.2ml. acetate

buffer layered on to a 4.6ml. 3-20% linear sucrose density gradient in 0.01M-sodium acetate buffer (pH 5.25) which contained 0.05M-NaCl. Gradients were centrifuged for 3 hr. at 125,000g in a Spinco model L ultracentrifuge (S.W.39 rotor).

The bottom of each gradient tube was then pierced with a syringe needle, 15 drop samples collected and 3ml. water added to each for ultraviolet absorption measurements.

Radioactivity measurements were carried out on 2ml. aliquots added to 8ml. dioxane containing Scinstant NE 572 (Nuclear Enterprises Ltd., Edinburgh) using a Nuclear Chicago liquid scintillation spectrometer.

Experiments on Isolated Nuclei

Preparation of Sheep-Thyroid Nuclei

(a) "Sucrose" nuclei. Nuclei were prepared from about 20g. sheep thyroid tissue as shown in outline in Fig. 15. All extraneous material was removed from the glands and the tissue minced twice at 0°, first using an ordinary mincer and then using a Latapie mincer. This stage was necessary prior to homogenization since thyroid tissue is very fibrous and tough and vigorous treatment during homogenization would damage the

Fig. 15

ISOLATION OF THYROID NUCLEI

Thyroid glands were minced and homogenized in ice-cold 0.25M-sucrose containing MgCl_2 and CaCl_2 . Homogenate was filtered through muslin and centrifuged at 0° for 5 min. at 100g.

Sediment
(discard)

Supernatant

Centrifuged over an equal volume of cold 0.35M-sucrose containing MgCl_2 and CaCl_2 at 1,000g for 5 min. at 0° .

Supernatant
(discard)

Sediment

Resuspended in a small volume of 0.25M-sucrose containing MgCl_2 and CaCl_2 , mixed with 19 volumes cold 2.3M-sucrose containing MgCl_2 and CaCl_2 and centrifuged at 0° for 1 hr. at 40,000g.

Supernatant
(discard)

Sediment
of yellow-white
coloured nuclei.

nuclei. The minced tissue was then homogenized in 10 volumes ice-cold 0.25M-sucrose containing 0.001M-MgCl₂ and 0.002M-CaCl₂ using a teflon-glass homogenizer of the Potter-Elvehjem type. After filtering through a double layer of muslin, the debris and unbroken cells were removed by centrifugation at 100g for 5 min. at 0°. The supernatant was layered on to an equal volume of cold 0.35M-sucrose containing 0.001M-MgCl₂ and 0.002M-CaCl₂ in centrifuge tubes and a crude nuclear fraction spun down at 1,000g for 10 min. at 0°. The nuclear pellets were resuspended in a small volume of cold 0.25M-sucrose containing 0.001M-MgCl₂ and 0.002M-CaCl₂, mixed with 19 volumes cold 2.3M-Sucrose containing 0.001M-MgCl₂ and 0.002M-CaCl₂ and centrifuged at 40,000g for 1 hr. at 0° to obtain pellets of clean nuclei. After rinsing the centrifuge tubes with cold 0.25M-sucrose to remove other material present, the nuclei were washed once and re-suspended in 0.25M-sucrose. Nuclei prepared by this method were used for the in vitro studies described later in this section.

(b) "Citric Acid" nuclei. These were prepared from about 20g. sheep-thyroid tissue as follows. The thyroid tissue was cleaned and minced twice as described above and homogenized in

20 volumes ice-cold 0.025M-citric acid containing 0.1% bentonite. After filtering through several layers of muslin, the homogenate was centrifuged at 1,500g for 5 min. at 0°. The pellets obtained from this spin were resuspended in 10 volumes 0.025M-citric acid and centrifuged at 1,500g for 5 min. at 0°. The pellets of nuclei obtained were drained free of acid and used for the isolation of purified sheep-thyroid nuclear RNA.

Incubation Conditions for In Vitro Studies using Isolated Nuclei

Studies using purified nuclei were carried out using the incubation mixture described by Allfrey, Mirsky and Osawa (1957), the pH of incubation being 7.1. Details of the incubation medium are shown in Table 10. Sheep-thyroid nuclei containing about 400µg. DNA (Ceriotti, 1952) were added to the incubation medium (total volume 1ml) which also contained 1µc (8-¹⁴C) adenine for studies of incorporation into RNA or 1µc (1-¹⁴C) DL-leucine for studies of incorporation into protein. In all cases the nuclei were added last to the incubation mixture to start the reaction, the samples incubated for various lengths of time from 0 to 60 min. at 37° with continuous shaking, and the reactions terminated by plunging the sample tubes into a freezing mixture (solid CO₂

Table 10

NUCLEAR INCUBATION MIXTURE

(according to Allfrey, Mirsky and Osawa, 1957)

Aliquots of nuclear suspension were incubated at 37° with continuous shaking in the presence of isotopically-labelled substance, buffer and added metabolites as follows:-

- (1) 0.3ml. nuclear suspension in 0.25M-sucrose.
- (2) 0.25ml. 0.1M-sodium phosphate buffer (pH 7.1) containing 0.25M-sucrose.
- (3) 0.2ml. 0.1M-glucose solution containing 3.75mg. NaCl and 5.34mg. $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$ per ml.
- (4) 0.05ml. water containing radioactive isotope e.g. (^{14}C) adenine.
- (5) 0.2ml. 0.25M-sucrose containing any additions to the medium e.g. TSH.

Total Volume = 1ml.

- ethanol). Zero time controls were also prepared in each case and the values obtained, which were always low, were subtracted from the values of the incubated samples. In one series of experiments the effect of omitting glucose from the incubation medium on the uptake of (^{14}C) adenine into nuclear RNA was studied. The effects of adding various compounds, such as TSH, puromycin and actinomycin D, to the incubation medium on the incorporation of labelled precursors into both RNA and protein were also investigated. In some cases the uptake of labelled precursors into the RNA and protein of sheep-thyroid nuclei was compared with that into rat-liver nuclei. For this purpose rat-liver nuclei were isolated using a procedure (Munro, Waddington and Begg, 1965) similar to that employed for the preparation of sheep-thyroid nuclei.

Separation of Nuclear RNA and Protein Fractions for Estimation and Radioactivity Measurement

After thawing the nuclear samples to 0° , they were treated as described earlier in this section for the tissue-slice fractions using the method of Fleck and Munro (1962) to extract RNA and protein. In these experiments it was not necessary to

add carrier albumin to co-precipitate the nucleic acids as there was sufficient protein present in the nuclear samples. The specific activities of the RNA fractions were determined as described previously except that when (32 -P) UTP was used as the RNA precursor, the samples were precipitated with cold HClO_4 containing UTP (2mg/ml) in place of adenine. For specific activity determinations on nuclear protein samples, the residues of protein and DNA remaining after RNA extraction were dissolved in 0.5ml. 0.3N-KOH by incubation at 37° for 1 hr. Aliquots (0.2ml) were then plated on to metal planchettes and counted as described earlier for RNA samples. Samples (0.1ml) were diluted to 1ml. with water and protein estimations carried out by the method of Lowry et al. (1951) using bovine serum albumin as standard. The specific activities of the nuclear protein samples were then calculated in terms of counts/min./mg. protein.

DNA Estimations on Sheep-Thyroid Nuclei

These were carried out on samples of the DNA fractions prepared by the method of Fleck and Munro (1962) using the procedure of Ceriotti (1952). This method gives satisfactory estimates of nuclear DNA since there is no thyroglobulin present

which interferes in the colorimetric reaction (see Fig. 9).

Preparation of Purified Sheep-Thyroid Nuclear RNA and Analysis on Sucrose Density Gradients

RNA was prepared from sheep-thyroid nuclei which had been isolated by either of the two methods described earlier. Samples of nuclei were homogenized in 5ml. 0.05M-tris-HCl buffer (pH 7.4) containing 1% sodium dodecyl sulphate and 10mg% bentonite. This gave a very viscous solution which was then shaken with an equal volume of 90% (w/v) phenol containing 0.1% 8-hydroxyquinoline for 3 min. at 60°. After cooling in ice, the aqueous phase was separated by centrifugation at 30,000g for 15 min. Bentonite was added giving a final concentration of 10mg% to the aqueous phase which was then extracted 3 times with an equal volume of ether. The aqueous phase was made 2% with respect to potassium acetate and the RNA precipitated with 2 volumes ethanol at -10° for 1 hr. The precipitate was centrifuged down at 2,000g for 15 min. at 0° and the pellet resuspended in 3ml. 0.05M-tris-HCl buffer (pH 7.4) containing 0.001M-MgCl₂ and 10mg% bentonite. Pancreatic DNase (5µg/ml) was added and the samples incubated for 5 min.

at 37⁰, then recooled in ice. The RNA was then reprecipitated with a final concentration of 25% ethanol and 2M-potassium acetate at -10⁰ for 1 hr. The RNA precipitate was centrifuged down at 2,000g for 15 min. at 0⁰ and resuspended in 0.5ml. 0.01M-sodium acetate buffer (pH 5.25) containing 0.05M-NaCl, and the insoluble material such as bentonite removed by centrifugation.

Ultraviolet absorption measurements were carried out to determine the amount of RNA present and the concentration adjusted to about 600µg/ml. About 100µg. RNA were then applied to 4.6ml. of a 3-25% linear sucrose gradient in 0.01M-sodium acetate buffer (pH 5.25) containing 0.05M-NaCl and centrifuged for 3 hr. at 125,000g in a Spinco model L ultracentrifuge (S.W.39 rotor). The ultraviolet absorption of the gradient at 260mµ was recorded automatically by a flow-cell device in conjunction with a Spectronic 505 spectrophotometer.

Measurement of Dephosphorylation of UTP by Isolated Sheep-Thyroid Nuclei

Nuclear incubations were carried out as described previously using TSH at a concentration of 0.1 i.u./ml, 0.04 µmoles

-112-

(β - 32 P) UTP (7×10^6 counts/min./ μ mole) replacing the labelled adenine. Samples were incubated for 0 and 15 min. at 37° and the reactions stopped by rapid freezing. On thawing to 0° , the samples were precipitated with cold HClO_4 to a final concentration of 0.2N with respect to acid, and the precipitates washed twice with cold 0.2N - HClO_4 combining the supernatants in each case. These combined supernatants, the acid-soluble fractions, were then neutralised with KOH at 0° and the precipitates of KClO_4 centrifuged down. Aliquots (0.1ml) of the supernatant fluids obtained were then used for paper chromatographic separation of the nucleoside mono-, di- and triphosphates and inorganic phosphate. The method used was that described by Smith (1960) in which the components are separated by 1-dimensional chromatography on Whatman No. 1 paper using an isobutyric acid-water-ammonia-versene solvent at pH 4.6 (Krebs and Hems, 1952). Markers of UTP, UDP, UMP and inorganic phosphate were also prepared and the chromatograms run in ascending fashion for 11 hr. After drying, the spots of the nucleotides were detected using ultraviolet light and those of inorganic phosphate by staining (Smith, 1960). The chromatograms were then cut into strips, 2.5cm.wide, for

radioactivity measurements by scanning the strips for ^{32}P
at a rate of 0.1 cm/min. using a Packard radiochromatogram
scanner (model 7201).

RESULTS AND DISCUSSION

Experiments using Sheep-Thyroid Slices

Uptake of (^{14}C) Adenine into Thyroid Slice RNA

In these experiments consistent labelling between duplicate tissue slice samples of the same thyroid preparation was obtained but between different batches of thyroid tissue there were wide variations in activity. Fig. 16 shows the uptake of (^{14}C) adenine into the RNA of sheep-thyroid slices at various times of incubation up to 3 hr. In agreement with the work of Hall (1963), addition of TSH (0.1 i.u./ml) at the start of incubation stimulated the uptake of adenine into RNA over the 3-hr. period. However this increase in adenine uptake into RNA was not apparent to any extent during the first hr. of incubation. This experiment was then repeated taking measurements at 0.5 hr. intervals over the 3-hr. incubation period, followed by separation of each tissue sample into a nuclear and a cytoplasmic fraction at the end of incubation. Fig. 17 shows the uptake of (^{14}C) adenine into nuclear and cytoplasmic RNA over the 3-hr. period. Addition of TSH at the start of incubation had no effect on adenine incorporation

^{14}C - ADENINE UPTAKE BY THE RNA OF SHEEP -
THYROID SLICES.

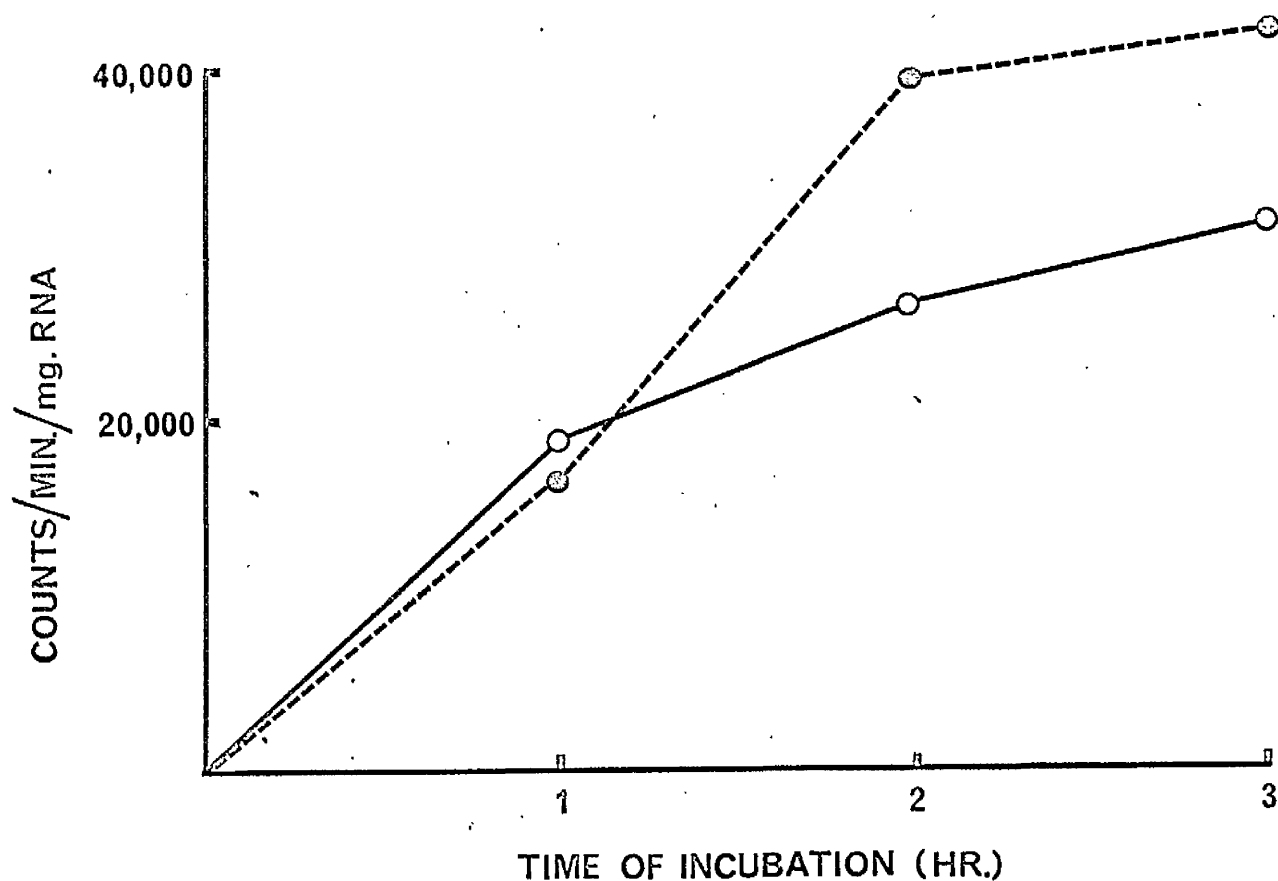


Fig. 16

Uptake of (^{14}C)-adenine by the RNA of sheep-thyroid slices during in vitro incubation in the presence or absence of TSH (0.1 i.u./ml.). Each point is the mean result of two experiments.

C-Adenine Uptake by
Thyroid Slices.

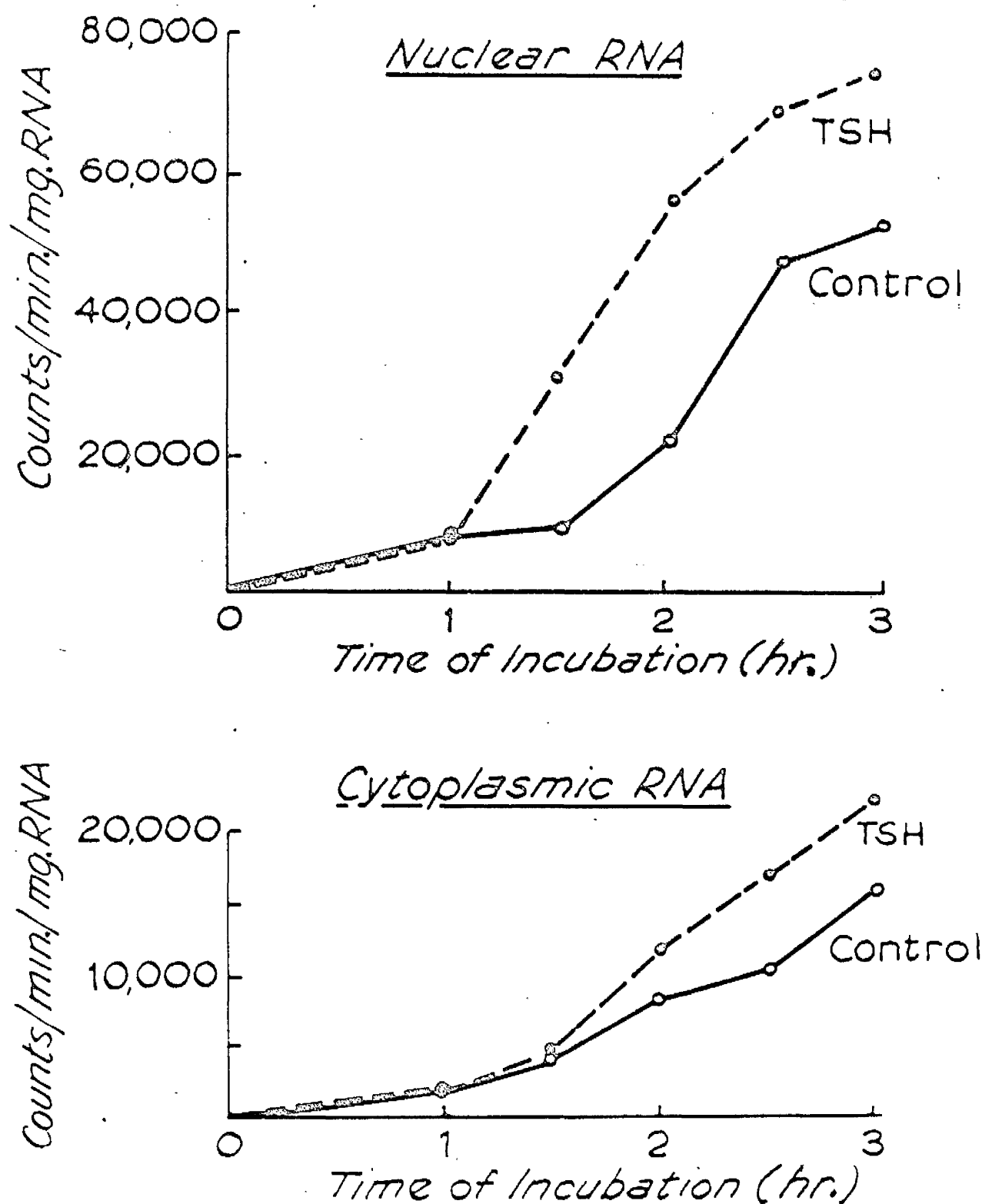


Fig. 17

Uptake of (^{14}C) adenine by the RNA of thyroid cell nuclei (upper graph) and cytoplasm (lower graph) during in vitro incubation of slices of sheep-thyroid glands in the presence or absence of TSH (0.1 i.u./ml.). Each point is the mean result of two experiments.

into RNA until 1 hr. had elapsed when an increase in uptake into nuclear RNA commenced. However the action of TSH on RNA labelling was not apparent in the cytoplasmic fraction until after 2 hr. incubation; a finding compatible with transfer of RNA from nucleus to cytoplasm.

Effects of Inhibitors on (^{14}C) Adenine Uptake into RNA
by Thyroid Slices

The effects of inhibitors of protein synthesis (puromycin) and DNA-dependent RNA synthesis (actinomycin D) were then examined in this system (Tables 11 and 12). Puromycin was added at a concentration ($100\mu\text{g/ml}$) which is known to inhibit uptake of (^{14}C) leucine into the protein of thyroid slices (Field, Johnson, Kendig and Fastan, 1963). When this inhibitor was added at the start of incubation it reduced the uptake of (^{14}C) adenine into nuclear RNA by about 60% and eliminated the increase in adenine uptake due to TSH (Table 11). When this inhibitor was added after 1 hr. of incubation, it still reduced (^{14}C) adenine incorporation, but TSH added at the start of incubation was now effective in stimulating adenine uptake into RNA. Similar results were obtained when the puromycin was

Table 11

Adenine Uptake by Nuclear RNA during
In Vitro Incubation of Thyroid Slices with
(¹⁴C) Adenine and TSH over a 3-hr. Period

<u>Time of adding inhibitor</u>	<u>(¹⁴C) Adenine Uptake (counts/min./mg. RNA)</u>		
	<u>Control Slices</u>	<u>TSH-treated Slices</u>	<u>Increase</u>
<u>Puromycin Addition:</u>			
None	53,500	75,300	+21,800
At 0 hr.	21,600	16,300	0
At 1 hr.	25,400	45,200	+19,800
At 2 hr.	53,900	74,000	+20,100
<u>Actinomycin D Addition:</u>			
None	14,200	18,200	+4,000
At 0 hr.	2,200	2,100	0
At 1 hr.	6,700	7,200	+500
At 2 hr.	99,200	9,600	+400

The hormone (0.1 i.u./ml.) was added at the start of incubation. Puromycin (100µg./ml. medium) and actinomycin D (10µg./ml. medium) were added at various times during the course of the 3-hr. incubation. The figures are the mean results of two experiments carried out in duplicate with each inhibitor.

Table 12

Adenine Uptake by Cytoplasmic RNA
during In Vitro Incubation of Thyroid Slices
with (^{14}C) Adenine and TSH over a 3-hr. Period

<u>Time</u> <u>of adding</u> <u>inhibitor</u>	<u>(^{14}C) Adenine Uptake (counts/min./mg. RNA)</u>		
	<u>Control</u> <u>Slices</u>	<u>TSH-treated</u> <u>Slices</u>	<u>Increase</u>
<u>Puromycin Addition:</u>			
None	16,200	22,600	+6,400
At 0 hr.	3,400	4,400	+1,000
At 1 hr.	5,200	9,300	+4,100
At 2 hr.	9,100	11,500	+2,400
<u>Actinomycin D Addition:</u>			
None	1,830	2,100	+270
At 0 hr.	660	660	0
At 1 hr.	970	980	+10
At 2 hr.	1,000	1,250	+250

The hormone (0.1 i.u./ml.) was added at the start of incubation. Puromycin (100 $\mu\text{g.}/\text{ml.}$ medium) and actinomycin D (10 $\mu\text{g.}/\text{ml.}$ medium) were added at various times during the course of the 3-hr. incubation. The figures are the mean results of two experiments carried out in duplicate with each inhibitor.

added after 2 hr. incubation, and the pattern of adenine incorporation into cytoplasmic RNA in general followed that into nuclear RNA apart from the value obtained when puromycin was added at the start of incubation (Table 12). The inhibition of TSH action with puromycin suggests that the mechanism involves synthesis of protein during the 1-hr. period before the increased (^{14}C) adenine uptake commences. However, it should be noted in this connection that Raghupathy, Abraham, Kerkof and Chaikoff (1964) showed that TSH had no detectable effect on the uptake of (^{14}C) leucine into the protein of sheep-thyroid slices in vitro at this time interval, and thus TSH under these experimental conditions cannot have a general action on thyroid protein synthesis.

Addition of actinomycin D ($10\mu\text{g/ml}$) at the start of incubation greatly reduced (^{14}C) adenine incorporation and inhibited the increased adenine uptake into RNA caused by TSH (Tables 11 and 12). When added after 1 hr. of incubation, the effect of this inhibitor on adenine uptake into RNA was still apparent; a small stimulatory action of TSH on adenine uptake was however obtained. Similar results were obtained when the

actinomycin D was added after 2 hr. incubation and again uptake of adenine into cytoplasmic RNA followed that into nuclear RNA (Table 12).

Sucrose Density Gradient Analysis of Thyroid Slice RNA

RNA was prepared from whole unfractionated slices (control and TSH-treated) which had been incubated for 3 hr. with (^{14}C) adenine. The RNA from each sample was fractionated on a sucrose density gradient and radioactivity measurements carried out (Fig. 18). Fig. 18 shows the results of one of three experiments all of which gave the same pattern of results. TSH appears to cause an increase in (^{14}C) adenine uptake into all species of RNA over a 3-hr. incubation period. From the absorption profile at 260m μ of the tissue-slice RNA it can be seen that the RNA is partially degraded particularly in the region of the heavier ribosomal peak, which is small compared with the lighter ribosomal peak, and this results in a corresponding increase in E_{260} in the region of sRNA. Also the (^{14}C) labelling in the region of sRNA was always great. This is probably due in part to degradation of heavier RNA and also to terminal -CCA turnover of thyroid tRNA. From examination of the absorption profiles

EFFECT OF TSH ON THE UPTAKE OF ^{14}C -ADENINE
INTO RNA BY SHEEP THYROID SLICES IN VITRO.

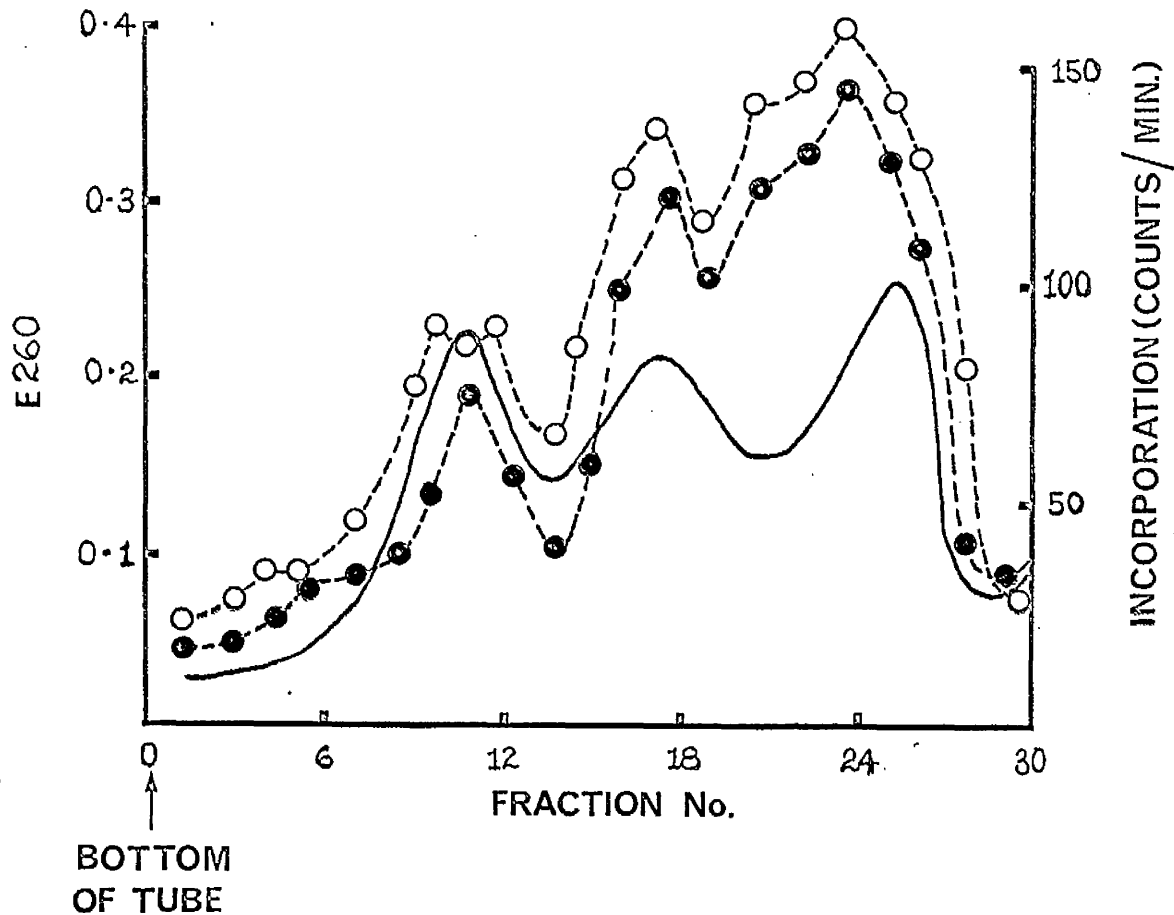


Fig. 18

Sucrose density gradient analysis of RNA prepared from thyroid slices which had been incubated with (^{14}C) adenine in the presence or absence of TSH for 3 hr. at 37° . The continuous line represents the E_{260} for the control samples which was very similar in pattern and position to that of the TSH-treated samples. The dotted lines show the (^{14}C) activity/fraction for each type. ○, TSH -treated; ●, control.

at various times throughout incubation, it was found that part of the degradation occurred during preparation of the tissue slices, the remainder occurring during incubation. At early times of incubation it was impossible to detect significant differences in labelling of RNA due to TSH since most of the labelling occurred near the top of the gradient, probably due in part to degradation of heavier RNA.

In summary, incubation of tissue slices of sheep thyroid with TSH and (^{14}C) adenine shows an increased labelling of nuclear RNA and subsequently of cytoplasmic RNA (Fig. 17), and this increase is apparently present after 3 hr. in all thyroid RNA fractions (Fig. 18). This effect of TSH can be inhibited by puromycin or actinomycin D added along with the TSH to the slices, and in consequence it appears that the action of TSH on thyroid RNA metabolism involves synthesis of protein and of RNA.

Studies using Purified Sheep-Thyroid Nuclei

Isolation of Nuclei

At present there are no published data available on methods of preparation of thyroid nuclei which are metabolically

active. From an examination of reviews of procedures available for the isolation of nuclei from other mammalian tissues (Siebert and Smellie, 1957; Allfrey, 1959; Roodyn, 1963) it would appear that a modification of the method of Chauveau, Moulé and Rouiller (1956) involving purification by centrifugation through dense sucrose would be the most suitable for preparing thyroid nuclei free from cytoplasmic contamination without great losses of nuclear material. The nuclear isolation procedure described in the "methods" section gave metabolically active nuclei in a yield of about 50% of the total population of nuclei in the thyroid as judged by the recovery of DNA from sheep-thyroid samples of known DNA content. Examination by light microscopy and by electron microscopy showed no evidence of significant contamination with whole cells or cytoplasmic components. Chemical analysis for RNA, DNA and protein gave a mean RNA/DNA ratio of 0.22 (0.20 - 0.26) and a mean protein/DNA ratio of 3.0 (2.7 - 3.3) over a series of experiments. The mean RNA/DNA ratio of sheep-thyroid nuclei prepared by the citric acid procedure was 0.20, a value slightly lower than that obtained using the sucrose procedure. There are no published

data available for these ratios in thyroid nuclei, but the results are in agreement with values obtained for nuclear preparations from other mammalian tissues such as liver (Roodyn, 1963).

In Vitro Studies

It has been shown by Allfrey, Mirsky and Osawa (1957) that isolated calf-thymus nuclei are able to incorporate labelled amino acids into protein and labelled RNA precursors into RNA. In vitro studies have also been carried out using isolated rat-liver nuclei, and there is evidence that they can accumulate adenine (Lee and Holbrook, 1964), synthesise ATP (Klouwen, Betel, Appelman and Arts, 1965) and synthesise RNA (Rees and Rowland, 1961). Isolated rat-liver nuclei are also able to incorporate isotopically-labelled amino acids into protein in vitro (Rendi, 1960; Rees and Rowland, 1961). The studies carried out on isolated sheep-thyroid nuclei demonstrate that they are also capable of in vitro synthesis of RNA and protein, and the results obtained during these experiments will now be described in detail. As in the tissue slice experiments, duplicate results of incorporation of labelled precursors into RNA or protein

obtained using nuclei from one batch of sheep-thyroid glands were in good agreement but variation in activity of nuclei prepared from different batches of thyroid tissue was quite great.

Uptake of Labelled Precursors into RNA by Isolated Nuclei

The effect of omitting glucose from the incubation medium on the uptake of (^{14}C) adenine into the RNA of sheep-thyroid nuclei over a 45-min. incubation period is shown in Fig. 19. It can be seen that glucose stimulates labelled adenine uptake into RNA under the incubation conditions used, possibly by acting as a source of energy for conversion of adenine to ATP for its subsequent incorporation into RNA.

The effect of TSH at various concentrations (0.01, 0.1 and 1.0 i.u./ml) on the uptake of (^{14}C) adenine into nuclear RNA was then studied over a 40-min. incubation period. Fig. 20 shows that the incorporation of labelled adenine is sensitive to the presence of TSH in the incubation medium. Increasing levels of TSH resulted in a stimulation of adenine incorporation into RNA which was roughly proportional to the logarithm of the dose added (Fig. 20). A TSH concentration of 0.1 i.u./ml was used throughout

^{14}C -Adenine Uptake by RNA of Incubated Thyroid Nuclei.

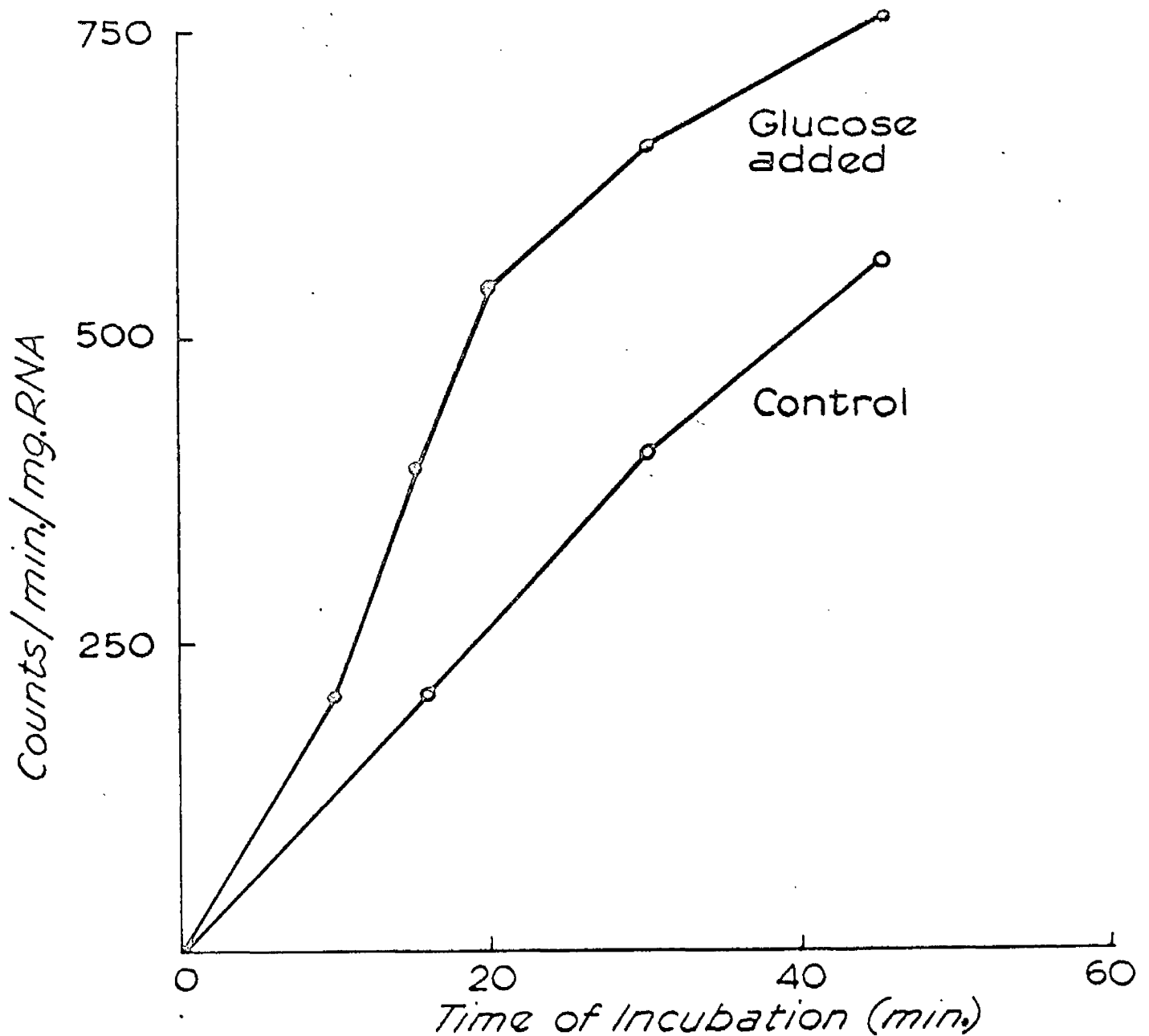


Fig. 19

Uptake of (^{14}C) adenine by the RNA of thyroid cell nuclei incubated in the presence or absence of glucose. Each point is the mean result of two experiments.

^{14}C -Adenine Uptake by RNA of
Incubated Thyroid Nuclei.

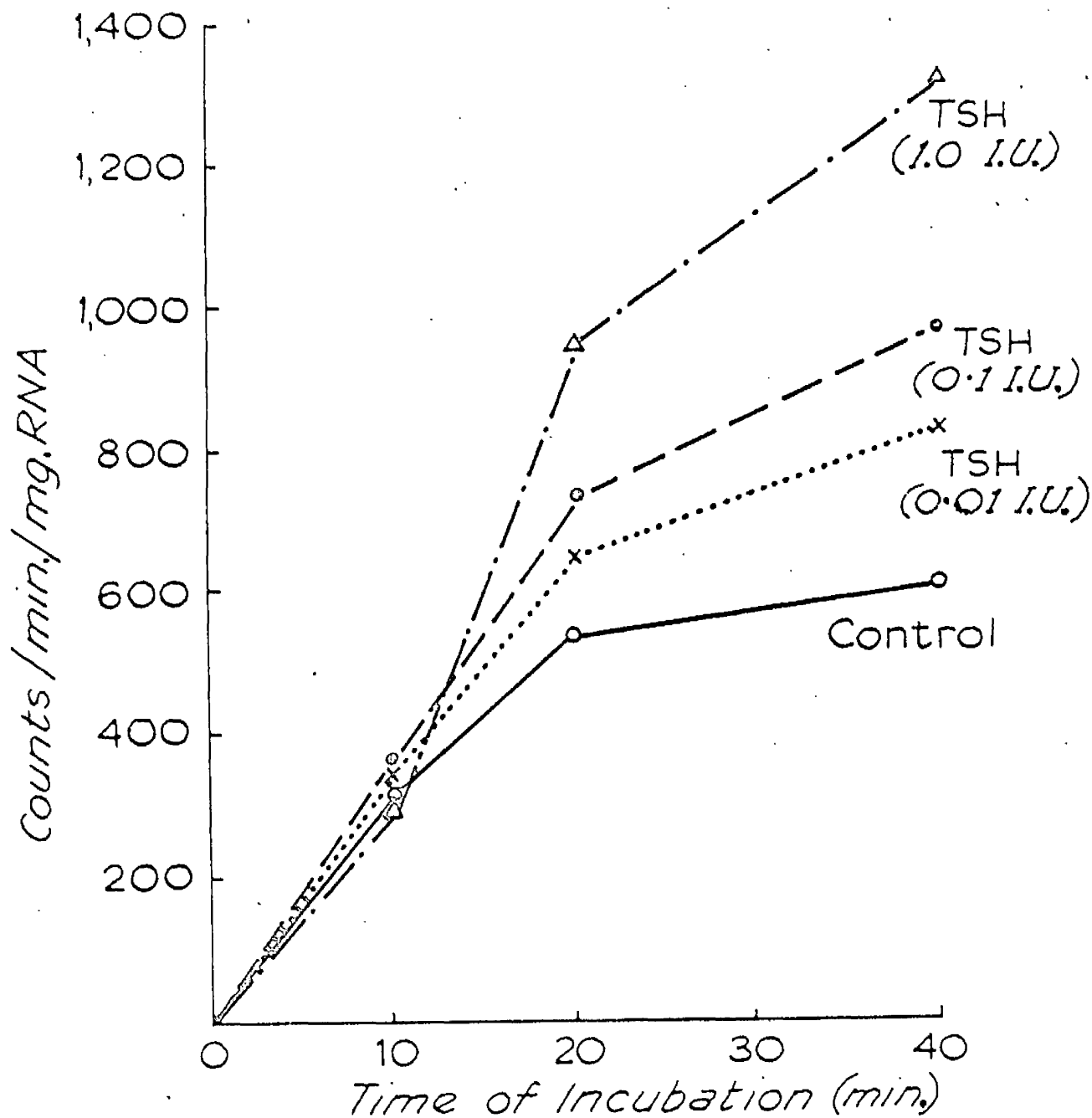


Fig. 20

Uptake of (^{14}C) adenine by the RNA of thyroid cell nuclei during incubation of isolated nuclei with TSH at various concentrations (0.01, 0.1 or 1.0 i.u./ml.). Each point is the mean result of two experiments performed in duplicate.

the remainder of the experiments and it was found that this concentration of TSH caused a significant increase in the uptake of (^{14}C) adenine into the RNA of sheep-thyroid nuclei ($P < 0.05$).

In one experiment sheep-thyroid nuclei were incubated with TSH or bovine serum albumin at a protein concentration ($100\mu\text{g/ml}$) equivalent to that of TSH (0.1 i.u./ml) along with control samples, and the uptake of (^{14}C) adenine into RNA again measured. Table 13 shows that addition of bovine serum albumin to the incubation medium had no effect on adenine uptake although the increase in uptake due to TSH was again apparent, and thus it appears that the TSH effect cannot be attributed to some non-specific reaction occurring on the addition of protein to the incubation medium. Also when rat-liver nuclei were incubated in the presence or absence of TSH under the same experimental conditions no increase in (^{14}C) adenine incorporation into rat-liver nuclear RNA was observed (Fig. 21). Thus the TSH effect does not appear to be a general one for isolated nuclei.

The increased incorporation of (^{14}C) formate into RNA adenine prepared from a crude nuclear fraction of calf-thyroid slices after incubation in the presence of TSH had been observed

Table 13

Effect of Bovine Serum Albumin or TSH on the
Uptake of (^{14}C) Adenine into RNA of
Sheep-Thyroid Nuclei In Vitro

<u>Incubation Time</u>	<u>(^{14}C) Adenine Uptake (counts/min./mg. RNA)</u>		
<u>(min.)</u>	<u>Control</u>	<u>Albumin-treated</u>	<u>TSH-treated</u>
20	426	428	635
30	787	819	990

Bovine serum albumin (100 $\mu\text{g.}$) or TSH (0.1 i.u.)
was added at the start of incubation.

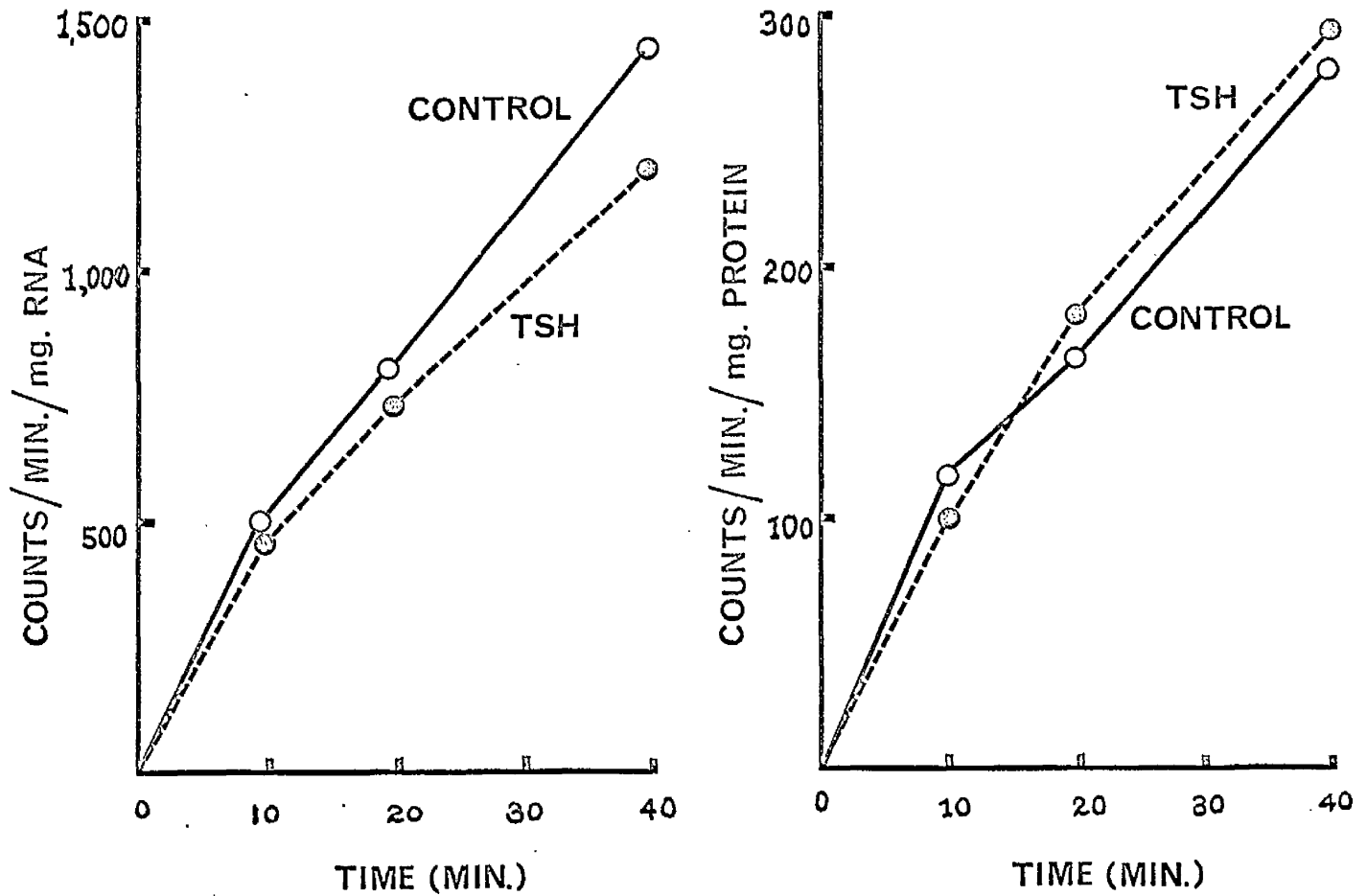


Fig. 21

Effect of TSH (0.1 i.u.) on the uptake of (^{14}C) adenine into RNA and (^{14}C) leucine into protein during incubation of isolated rat-liver nuclei. Each point is the mean result from two experiments.

by Hall (1963), who attributed this effect to more rapid formation of purine nucleotides for RNA synthesis. In order to determine whether the action of TSH on RNA synthesis resided in the pathway(s) leading from free base (e.g. adenine) to nucleoside triphosphate or on the subsequent synthesis of RNA from the nucleoside triphosphates, sheep-thyroid nuclei were incubated with (^{32}P) UTP (0.4 μmoles , 4×10^5 counts/min./ μmole) in place of the (^{14}C) adenine; unlabelled ATP, CTP and GTP were also added in equimolar proportions to the incubation mixture. Table 14 shows that TSH induced about the same increase in uptake of (^{32}P) UMP from the labelled UTP into RNA in the presence of the other nucleoside triphosphates as observed when nuclei were incubated with (^{14}C) adenine alone. Also the addition of the 4 nucleoside triphosphates (0.4 μmoles of each) to the incubation medium enhanced the incorporation of (^{14}C) adenine into nuclear RNA of both control and TSH-treated samples and decreased the increment in adenine uptake into nuclear RNA caused by TSH. Thus it would appear that TSH stimulates the synthesis of RNA from its nucleoside triphosphate precursors. However the

Table 14

Effect of TSH on Incorporation of Different RNA Precursors into Isolated Thyroid Nuclei Incubated in the Presence or Absence of Nucleoside Triphosphates

<u>Labelled Precursor</u>	<u>Addition of Triphosphates</u>	<u>Uptake of Label into RNA (counts/min./mg. RNA)</u>		
		<u>Control Nuclei</u>	<u>TSH-treated Nuclei</u>	<u>Difference</u>
(¹⁴ C) adenine	-	703	1,140	+437 (62%)
(¹⁴ C) adenine	+	982	1,308	+326 (33%)
(α - ³² P) UTP	+	280	473	+193 (69%)

Where indicated TSH (0.1 i.u.) and 0.4 μ moles each of ATP, CTP, GTP and UTP were added to the medium at the start of the 20-min. incubation period. When (α -³²P) UTP was used, unlabelled UTP was not added to the incubation medium. The figures are the mean results of two experiments, each performed in duplicate.

results do not eliminate the formation of ribonucleotides from the corresponding bases as a possible site of TSH action.

Uptake of (^{14}C) Leucine into Protein by Isolated Nuclei

The inhibition by puromycin of the action of TSH on RNA synthesis in thyroid slices (Table 11) suggests that the hormone may first affect the synthesis of nuclear protein. Thyroid nuclei were therefore incubated with (^{14}C) leucine in the presence or absence of TSH (0.1 i.u.) in order to test this hypothesis (Fig. 22). However it can be seen from Fig. 22 that the addition of TSH to the incubation medium had no significant effect on leucine incorporation into total nuclear protein, although the same dose increased the uptake of (^{14}C) adenine into thyroid nuclear RNA by about 60% over the same time interval (Fig. 20). Thus, under the experimental conditions used, TSH does not have a general effect on sheep-thyroid nuclear protein synthesis. Also when rat-liver nuclei replaced sheep-thyroid nuclei in the incubation, TSH had no effect on the uptake of (^{14}C) leucine into rat-liver nuclear protein (Fig. 21).

Addition of puromycin (100 μg) along with (^{14}C) leucine was found to inhibit the uptake of leucine into nuclear protein

^{14}C -Leucine Uptake by Protein of Incubated Thyroid Nuclei.

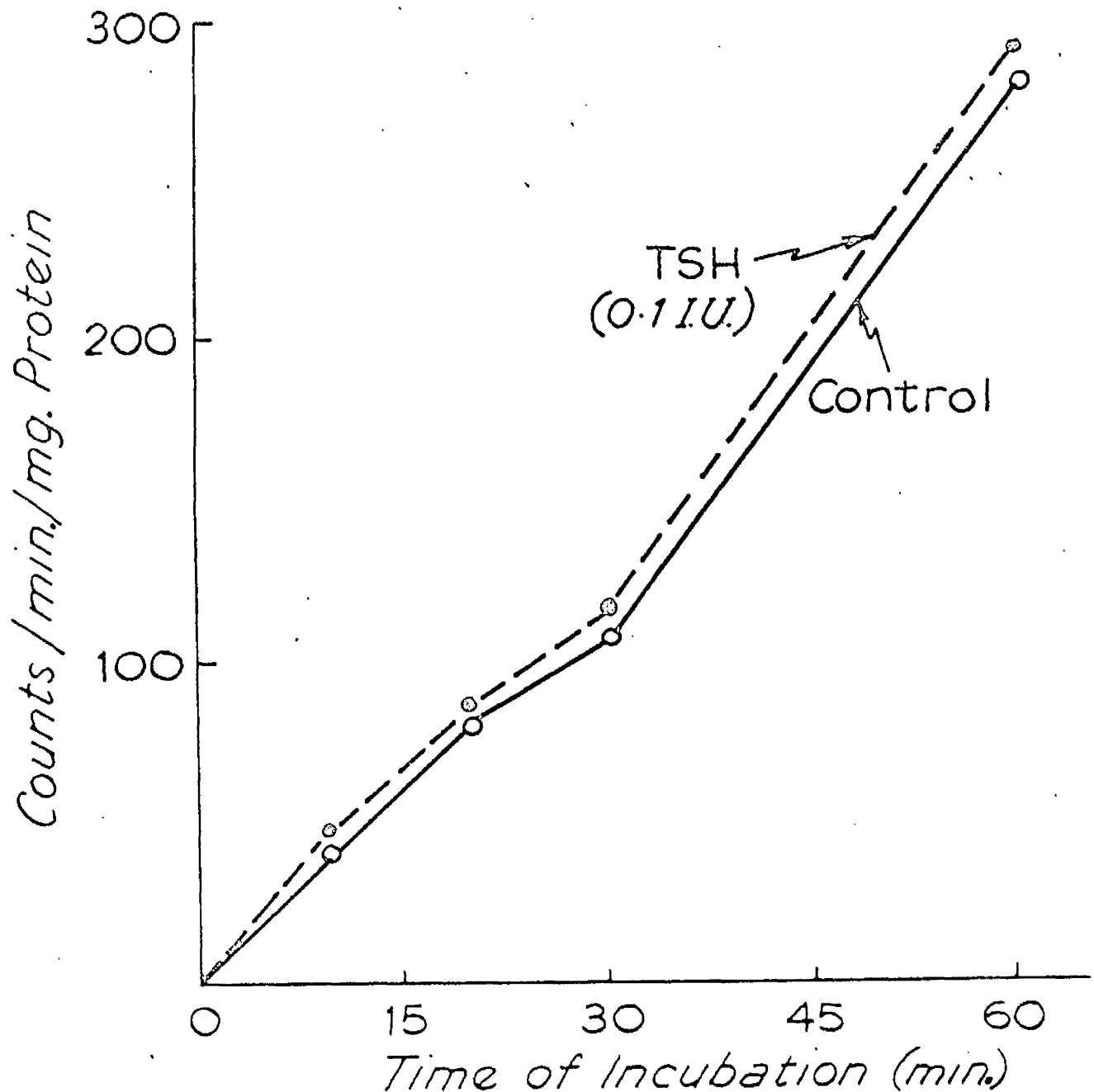


Fig. 22

Uptake of (^{14}C) leucine by the protein of thyroid cell nuclei during incubation of isolated nuclei with TSH at a concentration of 0.1 i.u./ml. Each point is the mean result of three experiments performed in duplicate.

by about 60% after 20 min. and by about 74% after 60 min. incubation in both control and TSH-treated nuclei (Table 15). The effect of actinomycin D (10 μ g) on the uptake of (14 C) leucine into thyroid nuclear protein was also investigated. The results obtained are shown in Table 15, and again no difference is apparent between the control and TSH-treated samples. Also it can be seen that actinomycin D unlike puromycin only inhibits the uptake of (14 C) leucine into nuclear protein by about 27% over a 60-min. incubation period.

Effect of Inhibitors on (14 C) Adenine Uptake into RNA by Sheep-Thyroid Nuclei

The action of puromycin and actinomycin D on RNA synthesis by isolated nuclei were investigated under two conditions of incubation. Puromycin or actinomycin D were added along with the (14 C) adenine and TSH, or else the nuclei were incubated with the inhibitors for 10 or 20 min. before the addition of label and hormone. Tables 16 and 17 show that the addition of puromycin (100 μ g) along with the (14 C) adenine and TSH did not affect incorporation into the RNA of the control nuclei at any time up to 30 min. incubation, nevertheless it depressed the action of added

Table 15

Effects of TSH and Puromycin or Actinomycin D on the Uptake of
(¹⁴C) Leucine into the Protein of Sheep-Thyroid Nuclei In Vitro

<u>Time of</u> <u>Incubation</u> <u>(min.)</u>	<u>(¹⁴C) Leucine Uptake into Protein (counts/min./mg. protein)</u>			
	<u>Control Nuclei</u>		<u>TSH-treated Nuclei</u>	
	<u>No</u> <u>Inhibitor</u>	<u>Puromycin</u> <u>Actinomycin D</u>	<u>No</u> <u>Inhibitor</u>	<u>Puromycin</u> <u>Actinomycin D</u>
10	41	28 31	48	30 33
20	81	35 61	80	32 71
30	110	44 90	119	54 98
60	286	75 200	277	79 208

TSH (0.1 i.u.), puromycin (100µg.) or actinomycin D (10µg.) were added to the medium at the start of incubation. The figures are the mean results of two experiments, each performed in duplicate.

Effect of Puromycin or Actinomycin D on (^{14}C) Adenine Uptake
by RNA during Incubation of Isolated Thyroid Nuclei In Vitro - I

Reincubation with Inhibitor (min.)	Time of Incubation (min.)	Control Nuclei			TSH-treated Nuclei			Increase with TSH		
		No Inhibitor	Puromycin	Actino- mycin D	No Inhibitor	Puromycin	Actino- mycin D	No Inhibitor	Puromycin	Actino- mycin I
None	10	330	580	470	840	1,130	830	+510	+550	+360
	20	1,020	1,200	930	2,520	2,000	1,390	+1,500	+800	+460
	40	1,800	1,780	1,040	2,900	2,040	2,100	+1,100	+260	+1,060
10	10	80	100	90	120	90	150	+40	0	+60
	20	520	260	260	730	310	420	+210	+50	+160
	40	750	630	400	1,500	730	460	+750	+100	+60

Puromycin (100 $\mu\text{g.}$) or actinomycin D (10 $\mu\text{g.}$) was added to the incubation mixture either at the time of addition of (^{14}C) adenine and TSH (0.1 i.e.) or at 10 min. beforehand. Each entry is the mean result of two experiments, and is expressed as counts/min./mg. RNA.

Effect of Puromycin or Actinomycin D on (14 C) Adenine Uptake
by RNA during Incubation of Isolated Thyroid Nuclei In Vitro - II

Incubation with Inhibitor (min.)	Time of Incubation (min.)	Control Nuclei			TSH-treated Nuclei			Increase with TSH		
		No Inhibitor	Puromycin	Actino- mycin D	No Inhibitor	Puromycin	Actino- mycin D	No Inhibitor	Puromycin	Actino- mycin D
None	10	400	400	370	890	840	520	+490	+440	+150
	20	1,030	1,240	780	2,900	1,540	1,900	+1,870	+300	+1,120
	30	1,670	1,440	960	3,400	1,650	2,320	+1,730	+210	+1,360
20	10	200	90	200	330	130	225	+130	+40	+25
	20	550	400	200	720	300	230	+170	+0	+30

Puromycin (100 μ g.) or actinomycin D (10 μ g.) was added to the incubation mixture either at the time of addition of (14 C) adenine and TSH (0.1 i.u.) or at 20 min. beforehand. Each entry is the mean result of two experiments, and is expressed as counts/min./mg. RNA.

TSH from 10 min. incubation onwards although puromycin only inhibits nuclear protein synthesis by about 60% at this time interval (Table 15). This confirms the conclusions drawn from the tissue-slice experiments (Table 11) that the increased incorporation of (^{14}C) adenine required preliminary synthesis of protein.

The addition of actinomycin D ($10\mu\text{g}$) to nuclei at the same time as addition of TSH and (^{14}C) adenine resulted in a depression of adenine uptake into the RNA of both control and TSH-treated nuclei throughout incubation (Tables 16 and 17). However it can be seen from Tables 16 and 17 that actinomycin D reduced the increased incorporation of adenine into nuclear RNA caused by addition of TSH only in proportion to its effect on adenine uptake by the RNA of the control nuclei. This indicates that actinomycin D added to the nuclei along with (^{14}C) adenine does not affect the increase in adenine uptake caused by TSH to a greater extent than its effect on general adenine uptake into nuclear RNA. This lack of effect of actinomycin D could be due to a greater rate of penetration and action of TSH added at the same time as the inhibitor. Accordingly,

nuclei were preincubated with actinomycin D or puromycin for 10 or 20 min. before the addition of TSH and (^{14}C) adenine. Tables 16 and 17 show that similar results were obtained at both time intervals, and that preincubation with either actinomycin D or puromycin inhibited the action of TSH on adenine incorporation. These findings suggest that the effect of TSH on nuclear RNA metabolism is both actinomycin D- and puromycin-sensitive, and thus may involve de novo synthesis of RNA and of protein.

Sucrose Density Gradient Analysis of RNA from Purified Sheep-Thyroid Nuclei

Absorption profiles at 260m μ of samples of RNA from sheep-thyroid nuclei prepared either by the sucrose or citric acid procedures are shown in Fig. 23. It can be seen from Fig. 23 that the RNA prepared from "sucrose" nuclei is partially degraded especially in the region of the heavier ribosomal component with a corresponding increase in RNA of smaller molecular weight in the region of sRNA. Also when RNA was isolated from "sucrose" nuclei which had previously undergone incubation at 37 $^{\circ}$ for 30 min., the RNA was found

EXTINCTION PROFILES AT 260m μ OF RNA PREPARED
FROM SHEEP-THYROID NUCLEI.

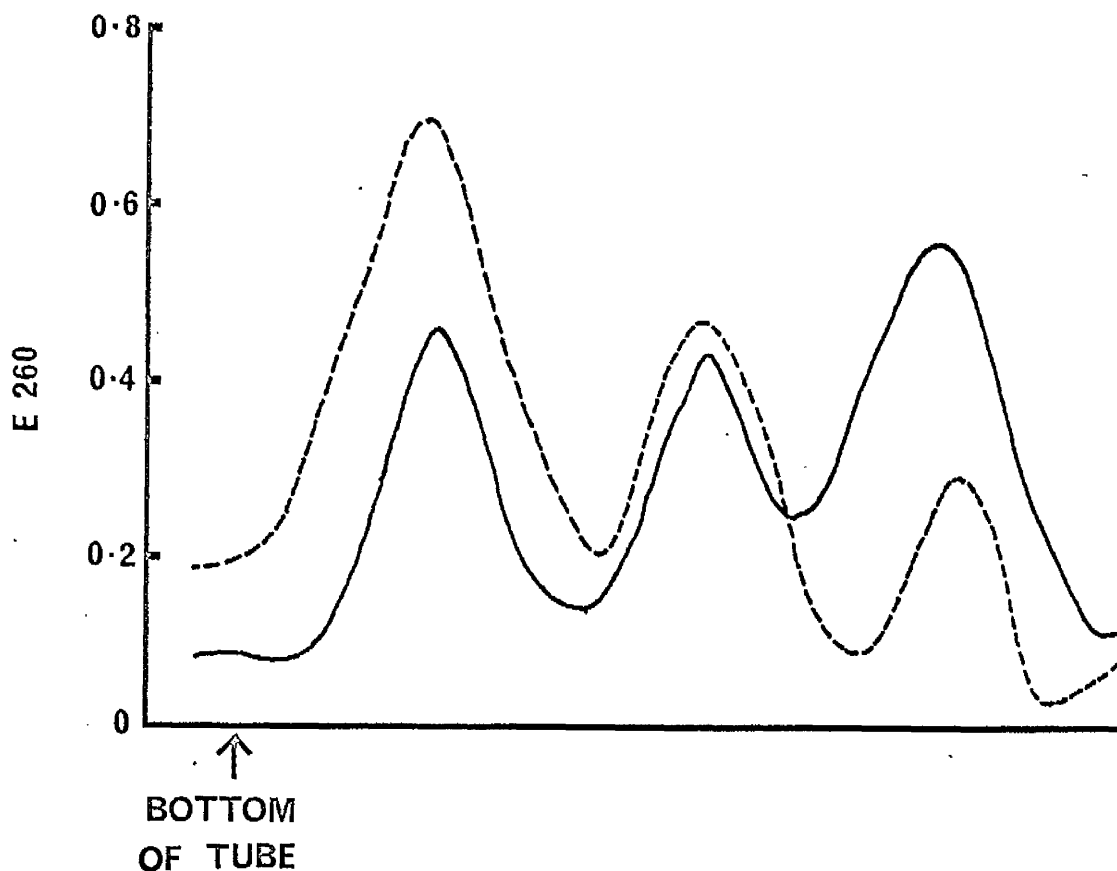


Fig. 23

RNA was prepared from sheep-thyroid nuclei which had been purified using either the sucrose or the citric acid procedure. Samples of RNA (100 μ g.) were layered on to 4.6 ml. linear sucrose density gradients (3-25%) and centrifuged for 3 hr. at 125,000 g . The ultraviolet absorption (at 260 m μ) of the gradients was then recorded automatically. The broken line represents the extinction profile of RNA prepared from "citric acid" nuclei and the unbroken line that from "sucrose" nuclei.

to be much more degraded with a very small amount of heavy RNA and an accumulation of degradation products in the region of sRNA. In contrast to the RNA prepared from "sucrose" nuclei, the RNA prepared from the "citric acid" nuclei was relatively undegraded as shown in Fig. 23. This is probably due to inhibition of ribonuclease activity by the acidic conditions used during nuclear isolation, whereas during the preparation and incubation of "sucrose" nuclei, ribonuclease was presumably not inhibited. Since the RNA from the "citric acid" nuclei was much less degraded, it might be thought that nuclei prepared by this method would be better for in vitro studies on RNA synthesis and turnover. This however is not the case since nuclei prepared by the citric acid procedure are not satisfactory for metabolic studies due to the acidic conditions employed, which damage the nuclei and also result in loss of acid-soluble nuclear material (Siebert and Smellie, 1957).

Ribonuclease Activity of Sheep-Thyroid Nuclei

"Sucrose" nuclei were incubated in the presence or absence of TSH (0.1 i.u.) for various lengths of time up to 1 hr. as described previously, and the RNA/DNA and protein/DNA ratios measured in each case. It was found that the RNA/DNA

ratio fell progressively throughout the period of incubation (from 0.22 to 0.18 over 1 hr.), whereas the protein/DNA ratio did not vary much (from 3.2 to 3.0 over 1 hr.) and there was no detectable difference between the control and TSH-treated samples. If it is assumed that DNA is not lost to any appreciable extent during incubation, the amount of RNA in isolated sheep-thyroid nuclei decreases by about 18% after in vitro incubation for 1 hr., and this degree of RNA loss is similar to that reported by Allfrey and Mirsky (1957) for calf-thymus nuclei. The nature of the enzymes responsible for this ribonuclease activity have not yet been characterized and no satisfactory means of inhibiting this activity was known.

UTP-Dephosphorylating Activity of Sheep-Thyroid Nuclei

It has been shown that rat-liver nuclei, purified by centrifugation through dense sucrose, contain enzymes which dephosphorylate ribonucleoside triphosphates in an ill-defined manner (Rees and Rowland, 1961; Reid, El-Aaser, Turner and Siebert, 1964; Siebert and Humphrey, 1965). The level of this activity was much greater than the release of pyrophosphate from nucleoside triphosphates during RNA synthesis. Thus the

dephosphorylating activity could affect RNA synthesis in in vitro systems using isolated nuclei by depleting the nuclei of RNA precursors in the form of nucleoside triphosphates, and also TSH could act by changing the rate of this process. This activity was therefore measured during in vitro incubations of sheep-thyroid nuclei. Incubations were carried out for 0 or 15 min. at 37° in the presence or absence of TSH (0.1 i.u.) to study the dephosphorylation of (^{32}P) UTP labelled in the $\beta\gamma$ phosphorus atoms. The results of the chromatogram scans of zero time and incubated samples are shown in Fig. 24. Each scan represents the mean of four samples incubated under identical conditions. It can be seen that sheep-thyroid nuclei possess considerable UTP-dephosphorylating activity, when comparison is made of the zero time sample and that incubated for 15 min., and that this is far in excess of the amount of UTP which is utilized during RNA synthesis. Also there was found to be no detectable difference between the control and TSH-treated samples. About 60% of the UTP present at the start of incubation was dephosphorylated over the 15-min. incubation period, and since 0.04 μmoles ($\beta\gamma$ - ^{32}P) UTP were initially

^{32}P ACTIVITY OF CHROMATOGRAMS OF THE PHOSPHATES OF
URIDINE AND INORGANIC PHOSPHATE ISOLATED AFTER
INCUBATION OF THYROID NUCLEI.

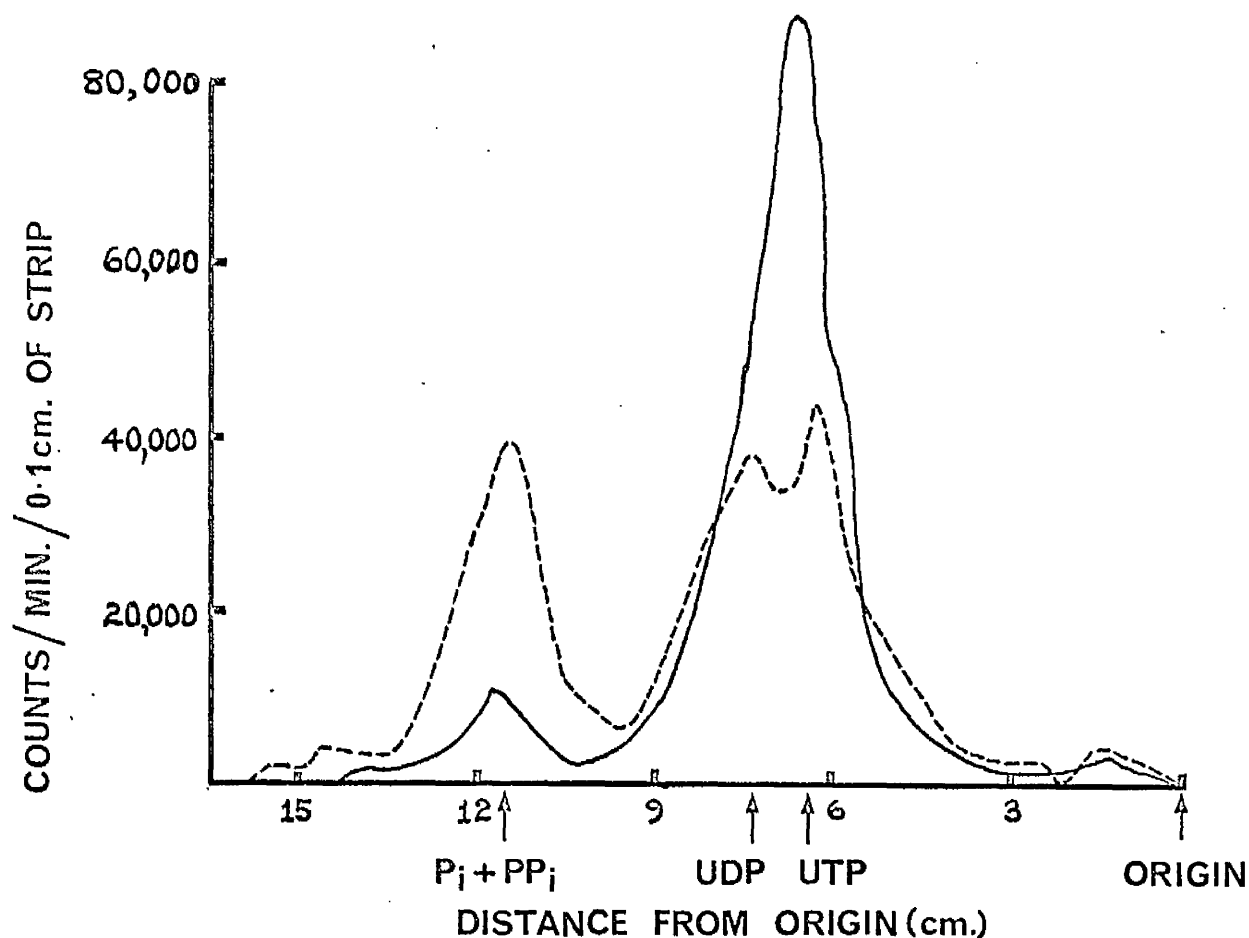


Fig. 24

Aliquots of sheep-thyroid nuclei were incubated with ($\beta\gamma$ - ^{32}P) UTP for 0 and 15 min. respectively. The acid-soluble fractions, containing the phosphates of uridine and inorganic phosphate, were then prepared and aliquots of each used for chromatography. The chromatograms were examined in ultraviolet light and stained for phosphate before scanning automatically for ^{32}P . The unbroken line represents the zero time sample, and the dotted line the sample which was incubated for 15 min.

present, this represents a dephosphorylation of 0.024 μ moles labelled UTP over the 15 min. period.

Comment on the Results obtained
using Isolated Nuclei

The in vitro studies carried out demonstrate that isolated sheep-thyroid nuclei are able to incorporate (^{14}C) leucine into protein and RNA precursors into RNA as do nuclei from calf-thymus or rat-liver tissue (Allfrey, Mirsky and Osawa, 1957; Roodyn, 1963). It was also found that the uptake of (^{14}C) adenine into RNA by isolated sheep-thyroid nuclei was sensitive to the presence of TSH in the incubation medium, the effect being roughly proportional to the logarithm of the amount of TSH added (Fig. 20). This stimulation of adenine uptake does not appear to be due to some non-specific reaction occurring on the addition of protein to the incubation medium since bovine serum albumin added at an equivalent protein concentration to the TSH hormone had no effect on the incorporation of labelled adenine into RNA (Table 13). Also rat-liver nuclei incubated under similar conditions were not responsive to TSH (Fig. 21). Thus sheep-thyroid nuclei appear to possess

some specialized mechanism whereby they can respond to the presence of TSH by altering their RNA metabolism.

Before accepting these findings as unequivocal, some possible fallacies must be considered. Since (^{14}C) adenine was used as the RNA precursor, it is always possible that TSH could cause the increase in incorporation into RNA by altering the rate of turnover of the three terminal -CCA nucleotides of tRNA without any effect on DNA-dependent RNA synthesis. However the experiments using actinomycin D, which inhibits DNA-dependent RNA synthesis but not -CCA terminal turnover of tRNA, rules out this possibility, and in addition TSH was found to increase the uptake of (α - ^{32}P) UTP into nuclear RNA. This latter observation is contrary to the postulate put forward by Hall (1963) that the effect of TSH on thyroid RNA metabolism is on the supply of available ribose for formation of RNA precursors. Also if this postulate explains the action of TSH on (^{14}C) adenine uptake, the addition of nucleoside triphosphates should eliminate the effect of TSH on adenine uptake by providing an excess of ready-made nucleotides. However, when nucleoside triphosphates were

added to the incubation medium, they decreased but did not abolish the TSH-dependent adenine uptake into RNA (Table 14) although they increased slightly the incorporation into the RNA of both control and TSH-treated samples. Also, when (α - 32 P) UTP was used as RNA precursor, the magnitude of the stimulation in uptake into RNA caused by TSH in the presence of equimolar amounts of ATP, CTP and GTP was similar to that obtained using only (14 C) adenine (Table 14). Sheep-thyroid nuclei can dephosphorylate UTP to a considerable extent by processes not involving RNA synthesis (Fig. 24) and thus the amount of (α - 32 P) UTP present in assays of uptake into RNA could be rate-limiting due to dephosphorylation. However the amount of (α - 32 P) UTP (0.4 μ moles) added to the incubation medium during studies of uptake into RNA was far in excess of the amount being dephosphorylated over the 20-min. incubation period as shown by the studies using (β - 32 P) UTP described above. Thus the amount of (α - 32 P) UTP remaining in the incubation medium could not have been rate-limiting during the studies on RNA synthesis.

It was found that RNA is being degraded to acid-soluble

products throughout the course of nuclear incubation with a loss of about 18% from sheep-thyroid nuclei over a 1-hr. period. From sucrose density gradient analysis of purified sheep-thyroid nuclear RNA (Fig. 23) it appears that the heavier ribosomal component is especially sensitive to degradation. Thus the measured uptake of labelled precursors into RNA of isolated sheep-thyroid nuclei will be the result of both the synthetic and degradative reactions of RNA occurring during incubation in vitro.

Uptake of (^{14}C) leucine into total nuclear protein was found to be unaffected by the presence of TSH in the incubation mixture (Fig. 22). However, this does not exclude an action of TSH limited to synthesis of one nuclear protein since the resulting increase in leucine incorporation would be too small to be detected under the conditions used. (^{14}C) leucine uptake into the protein of sheep-thyroid nuclei was found to be inhibited in the presence of puromycin by about 74% of the appropriate control samples incubated with or without TSH over a 1-hr. period (Table 15). Puromycin at the same concentration was found to inhibit leucine uptake into protein of calf-thymus nuclei by about 90% (Allfrey, Littau and Mirsky, 1964), and thus

puromycin does not appear to be as effective when added to isolated sheep-thyroid nuclei. The degree of inhibition of (^{14}C) leucine incorporation into nuclear protein caused by actinomycin D over the same time interval was found to be much smaller, about 27% (Table 15). Similar results for the effect of actinomycin D on the uptake of labelled amino acids into the protein of calf-thymus nuclei have been reported by Allfrey, (1963). The small degree of inhibition on actinomycin D treatment suggests that, after DNA-dependent RNA synthesis has ceased, there is sufficient messenger RNA available in the nuclei to code for protein synthesis for some time. Either nuclear messenger RNA is fairly stable when involved in nuclear protein synthesis, or else the normal cellular means of messenger RNA breakdown is non-functional. In connection with this, treatment with actinomycin D prior to nuclear isolation has been shown to inhibit ribonuclease activity in rat-liver nuclei (Siebert, Villalobos, Ro, Steele, Lindenmayer et al., 1966). It may be also that some of this messenger RNA is transferred in the intact cell to the cytoplasm where breakdown of the messenger can occur after it has coded for protein.

The effects of puromycin and actinomycin D on nuclear RNA metabolism were also investigated (Tables 16 and 17).

Puromycin added at the start of incubation with labelled adenine and TSH was found to inhibit the effect of added TSH on nuclear RNA metabolism from 10 min. incubation onwards. This is in agreement with results obtained from the tissue slice experiments (Table 11) namely that the increased incorporation of (^{14}C) adenine into RNA caused by TSH addition requires preliminary synthesis of protein. However, it was found that sheep-thyroid nuclei had to be preincubated with actinomycin D before the addition of label and hormone in order to inhibit the action of TSH on adenine uptake. The lack of immediate effect of actinomycin D could be due to a greater rate of penetration and action of TSH added at the same time as the inhibitor.

These findings suggest that the effect of TSH on nuclear RNA metabolism is actinomycin D-sensitive and thus may involve the synthesis of messenger RNA which perhaps could code for a specific nuclear protein. From the puromycin studies, it appears that synthesis of protein is also essential for the action of TSH on nuclear RNA metabolism. Since this protein is necessary for the

stimulatory effect of TSH on general thyroid RNA synthesis, it could perhaps be a DNA-dependent RNA polymerase (or polymerases).

SUMMARY

1. When tissue slices of sheep thyroid were incubated with TSH and (^{14}C) adenine, it was found that TSH caused an increase in labelling of nuclear RNA and subsequently of cytoplasmic RNA, and that this increase was detectable after 3 hr. incubation in all thyroid RNA fractions. This effect could be inhibited by puromycin and actinomycin D added along with TSH to the slices, and in consequence it appears that the action of TSH on thyroid RNA metabolism involves synthesis of RNA and protein.

2. A method has been devised for the isolation of purified sheep-thyroid nuclei in reasonable yield. Nuclei prepared by this method are capable of incorporating isotopically-labelled amino acids into protein and RNA precursors into RNA.

3. In Vitro Studies using Isolated Nuclei

(a) Uptake of Labelled Precursors into RNA Uptake of (^{14}C) adenine into the RNA of sheep-thyroid nuclei was reduced

when glucose was omitted from the incubation medium. The incorporation of (^{14}C) adenine into nuclear RNA was also found to be sensitive to the presence of TSH in the incubation medium. Increasing levels of TSH resulted in a stimulation of adenine uptake which was roughly proportional to the logarithm of the dose added. Rat-liver nuclei were not responsive to TSH under the same conditions of incubation, and addition of protein (bovine serum albumin) to the incubation medium had no effect on adenine uptake into sheep-thyroid nuclear RNA. TSH also stimulated the uptake of (α - ^{32}P) UTP into RNA in the presence of the other unlabelled ribonucleoside triphosphates, and addition of all four unlabelled ribonucleoside triphosphates to the incubation medium did not abolish the increase in adenine uptake into nuclear RNA caused by TSH.

(b) Uptake of (^{14}C) Leucine into Protein Addition of TSH to the incubation medium had no significant effect on (^{14}C) leucine incorporation into the protein of sheep-thyroid nuclei under incubation conditions where it stimulated (^{14}C) adenine uptake into nuclear RNA. Puromycin was found to inhibit the uptake of (^{14}C) leucine into thyroid nuclear protein by about

74% over a 1-hr. incubation period, whereas actinomycin D only inhibited leucine uptake by about 27% over the same time interval.

(c) Effects of Inhibitors on (^{14}C) Adenine Uptake into RNA of Isolated Sheep-Thyroid Nuclei Puromycin added at the start of incubation with (^{14}C) adenine and TSH was found to inhibit the effect of added TSH on nuclear RNA metabolism from 10 min. incubation onwards. It was found however that sheep-thyroid nuclei had to be preincubated with actinomycin D before the addition of label and hormone in order to inhibit the action of TSH on adenine uptake. The findings suggest that the earliest effects of TSH on nuclear RNA metabolism are both puromycin- and actinomycin D-sensitive and thus may involve the synthesis of messenger RNA which could then code for the synthesis of a specific nuclear protein.

(d) Ribonuclease Activity of Isolated Sheep-Thyroid Nuclei During in vitro incubation of isolated sheep-thyroid nuclei for 1 hr. at 37° about 18% of the total nuclear RNA is degraded to acid-soluble products. From sucrose density gradient analysis,

it was found that the heavier ribosomal RNA component appeared to be especially sensitive to degradation.

(e) UTP-Dephosphorylating Activity of Isolated Sheep-Thyroid Nuclei It was found that isolated sheep-thyroid nuclei like rat-liver nuclei contain enzymes which dephosphorylate UTP to a considerable extent in vitro. The level of this activity was found to be much greater than that releasing pyrophosphate from UTP during RNA synthesis.

SECTION III

**Studies on the DNA-dependent RNA Polymerase
Activity of Sheep-Thyroid Nuclei**

INTRODUCTION

Since the discovery of Weiss (1960) of a rat-liver nuclear enzyme that catalyses RNA synthesis by copolymerization of nucleotides derived from all four ribonucleoside triphosphates, evidence has accumulated that such RNA polymerases (nucleoside triphosphate: RNA nucleotidyl transferase E.C. 2.7.7.6) are largely if not solely responsible for the biosynthesis of all cellular RNAs which are complementary to specific regions of the DNA genome (Geiduschek, Nakamoto and Weiss, 1961; Robinson, Hsu, Fox and Weiss, 1964). In vivo administration of certain hormones such as androgens (Williams-Ashman, Liao, Hancock, Jurkowitz and Silverman, 1964), oestrogens (Gorski, 1964) and the thyroid hormones (Widnell and Tata, 1963) has been found to influence the DNA-dependent RNA polymerase activities of their target tissues. Such measurements have been made either on isolated cell nuclei or on "aggregate" enzyme preparations of the type described by Weiss (1960). The latter consist of nucleoprotein gels obtained by exposure of washed chromatin material to hypertonic KCl at low temperatures. The DNA that is firmly

bound to these mammalian nuclear extracts serves as the template for the associated RNA polymerases. The DNA-dependent RNA polymerases of certain bacteria have been extensively purified and completely separated from DNA (Chamberlain and Berg, 1962; Nakamoto, Fox and Weiss, 1964). In contrast, attempts to solubilize and purify the RNA polymerase from a number of types of mammalian cells and to free the enzyme from DNA have been generally unsatisfactory, and only in a few cases, e.g. chicken embryo (Furth and Loh, 1963) and rat testis (Ballard and Williams-Ashman, 1964), has it been possible to demonstrate RNA synthesis directed by exogenous DNA.

From the results described in Section II, it appears that TSH stimulates RNA synthesis in sheep-thyroid nuclei, and that synthesis of a small amount of nuclear protein is also necessary for this process. The protein which is affected by TSH and concerned in nuclear RNA synthesis could perhaps be an RNA polymerase. At present there is no information available on the DNA-dependent RNA polymerase activity of thyroid tissue, and therefore studies were carried out to characterize this enzyme

in sheep-thyroid, and to investigate the action of TSH on its activity.

MATERIALS AND METHODS

Chemicals used

ATP, CTP, GTP and UTP, bovine pancreatic DNase, puromycin, actinomycin D and (α - ^{32}P) UTP were obtained from the sources mentioned in Section II. Purified calf-thymus DNA and crystalline bovine pancreatic RNase were from the Sigma Chemical Co., St. Louis, Mo., U.S.A. TSH (1 i.u./mg. protein) was from the Armour Pharmaceutical Co., Illinois, and TSH (N.I.H.-TSH-B₃; 2.73 i.u./mg. protein) was a gift from the Endocrinology Study Section, N.I.H., Bethesda, Maryland. Ribonucleoside triphosphates labelled with ^3H or ^{14}C were from Schwarz Biochem Inc., Orangeburg, N.Y.

Preparation of DNA-Dependent RNA

Polymerase Enzyme Fractions

"Sucrose" nuclei were isolated from about 30g. sheep-thyroid tissue using the method described in Section II (Fig. 15), and were then either preincubated for 15 min. at 37° in the medium of Allfrey, Mirsky and Osawa (1957) without added

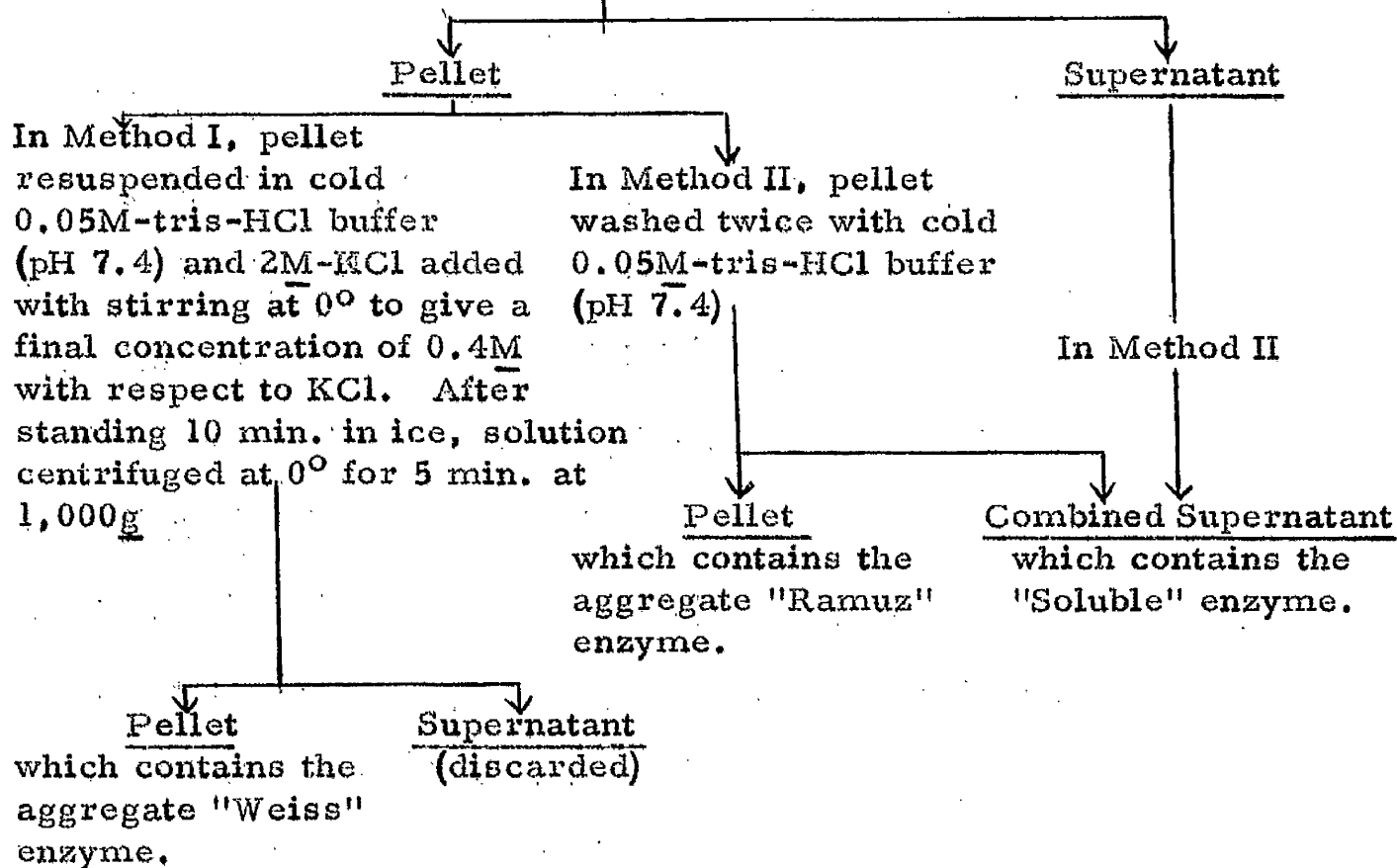
isotope as described in Section II or used directly for polymerase enzyme preparation. Enzyme preparations were carried out in two ways, and an outline of the procedures is shown in Fig. 25.

(1) Method according to Weiss (1960) The nuclei were suspended in 20 volumes ice-cold 0.05 M-tris-HCl buffer (pH 7.4) and allowed to stand 10 min. in ice. The lysed nuclei were then centrifuged at 10,000g for 10 min. at 0°, and the pellet obtained suspended by gentle homogenization in 4ml. 0.05M-tris-HCl buffer (pH 7.4) and 1 ml. 2M-KCl added dropwise to the suspension at 0° with adequate stirring. Within a few min. a white aggregate formed which could be collected as a pellet by centrifugation at 1,000g for 5 min. at 0°. The isolated aggregate was then washed twice in the tris-KCl medium and finally suspended in 5ml. cold 0.05M-tris-HCl buffer (pH 8.1) by homogenization.

(2) Method according to Ramuz, Doly, Mandel and Chambon (1965a) The nuclei were lysed as described above, and then centrifuged for 15 min. at 54,000g in a Spinco model L ultracentrifuge and both the pellet and the supernatant retained. The pellet was washed twice with a small volume ice-cold 0.05M-tris-HCl

Preparation of DNA-Dependent RNA Polymerase EnzymeFractions from Purified Sheep-Thyroid NucleiSheep-Thyroid Nuclei

Lysed in 0.05M-tris-HCl buffer (pH 7.4) for 10 min. at 0° then centrifuged at 0° for 10 min. at 10,000g in Method I, or for 15 min. at 54,000g in Method II.



buffer (pH 7.4) and the supernatant fractions combined. The pellet contained the "aggregate" enzyme, i.e. "Ramuz" enzyme, which was suspended by homogenization in a small volume cold 0.05M-tris-HCl buffer (pH 8.4). The supernatant fraction contained the "Soluble" enzyme. Before assaying the DNA-dependent RNA polymerase activity of the "Soluble" enzyme preparation, it was placed in a dialysis sac surrounded by polyethylene glycol and left for about 2 hr. at 4° to concentrate the protein.

RNA, DNA and Protein Estimations on the Enzyme Preparations

The protein concentration of the enzyme preparations was determined by the method of Lowry et al. (1951) using bovine serum albumin as standard. RNA and DNA were separated using the procedure of Fleck and Munro (1962), and estimations carried out as described for sheep-thyroid nuclei in Section II.

Assays of DNA-Dependent RNA Polymerase Activity

Composition of the Incubation Mixtures

(a) Using the "Weiss" enzyme preparation This was carried out using a modification of the method of Goldberg (1961). The

assay mixture contained: 50 μ moles tris-HCl buffer (pH 8.1), 1.5 μ moles MnCl_2 , 30 μ moles KCl, 2.5 μ moles 2-mercaptoethanol, 10 μ moles NaF, 0.4 μ moles each of ATP, CTP and GTP, 0.1 μ moles (α - ^{32}P) UTP (about 10^6 counts/min./ μ mole) or 0.05 μ moles (^{14}C) UTP (0.5 μc), 0.05ml. saturated $(\text{NH}_4)_2\text{SO}_4$ (adjusted to pH 8.1 with NH_4OH) if required, and 0.5mg. enzyme protein in a total volume of 0.5 ml.

(b) Using the "Ramuz" enzyme preparation The assay mixture contained: 50 μ moles tris-HCl buffer (pH 8.4), 1.5 μ moles MnCl_2 or 5 μ moles MgCl_2 , 30 μ moles KCl, 2.5 μ moles 2-mercaptoethanol, 0.4 μ moles each of the three unlabelled ribonucleoside triphosphates, 0.05 μ moles of the labelled ribonucleoside triphosphate (0.5 μc if ^{14}C -labelled and 2 μc if ^3H -labelled), 0.05ml. saturated $(\text{NH}_4)_2\text{SO}_4$ (pH 8.4) if required, and 0.5mg. enzyme protein in a total volume of 0.5ml.

(c) Using the "Soluble" enzyme preparation The assay mixture was similar to that described above for the "Ramuz" enzyme preparation except that the pH of the tris-HCl buffer was 7.2 and calf-thymus DNA (80 $\mu\text{g.}$) was present to serve as template for the enzyme reaction.

In some cases compounds, such as TSH and actinomycin D, were added to the assay mixtures. These were added dissolved in the appropriate tris-HCl buffer. The DNA-dependent RNA polymerase activities of the enzyme preparations were always assayed when the enzymes were freshly prepared. The enzymes were added last to start the reaction and the samples, which were usually present in duplicate, incubated for various lengths of time up to 40 min. at 37° with shaking. The reactions were stopped by rapid freezing in a mixture of solid CO₂ and ethanol, and at this stage, in some experiments, the samples were left frozen at -70° overnight.

Extraction of RNA and Radioactivity Measurements

On thawing the solutions to 0°, 0.1ml. carrier bovine serum albumin (20mg./ml.), 0.5ml. 0.1M-Na₄P₂O₇ and 1.1ml. ice-cold 0.4N-HClO₄ were added and the solutions mixed. (In later experiments 1.0ml. ice-cold 0.4N-HClO₄ containing 2% hyflo supercel (Hopkins and Williams Ltd., Essex) was used in place of 0.1ml. carrier albumin solution and 1.1ml. 0.4N-HClO₄). After standing 10 min. in ice, the samples

were centrifuged at 1,000g for 10 min. at 0°. To the precipitates, 0.3ml. 0.5N-NaOH was added followed rapidly by 0.5ml. 0.1M- $\text{Na}_4\text{P}_2\text{O}_7$ and 1.6ml. 0.4N- HClO_4 . After standing 10 min. in ice, the samples were again centrifuged at 1,000g for 10 min. and the precipitates obtained washed 3 times with 5ml. cold 0.2N- HClO_4 . The samples were then drained free of acid and digested for 1 hr. at 37° in 0.3N-KOH. After cooling the samples in ice, 0.5ml. ice-cold 2.4N- HClO_4 was added, and the precipitates of DNA and protein allowed to flocculate. The samples were then centrifuged at 1,000g for 10 min., and the precipitates obtained washed twice with 0.5ml. cold 0.2N- HClO_4 combining the supernatant fractions, which contained the acid-soluble RNA, in each case. 0.5ml. 0.3N-KOH was added to the precipitates which were then incubated for 1 hr. at 37° to solubilize the DNA and protein.

Aliquots (2ml.) of the acid-soluble RNA fractions were neutralised with KOH at 0° and the precipitates of KClO_4 centrifuged down. Samples of the supernatant fluids obtained were then used for radioactivity measurements. If the RNA had been labelled with ^{32}P , 0.5ml. samples were plated on to

metal planchettes using lens paper discs to give uniform self-absorption and counted in a Nuclear Chicago gas-flow counter. With RNA labelled with ^{14}C or ^3H , 2ml. samples were added to 10ml. dioxane containing Scinstant NE 572 (Nuclear Enterprises Ltd., Edinburgh) and counted in a Nuclear Chicago liquid scintillation spectrometer. The activity of the polymerase enzyme in each assay was then calculated in terms of μmoles labelled nucleotide incorporated into RNA/mg. protein present in the enzyme fraction.

Note: - The method of RNA isolation described above was used for several reasons. Firstly, it separates the RNA from DNA and protein and is known to give satisfactory recoveries of RNA (Fleck and Munro, 1962). It also separates the RNA from compounds, such as TSH, which were added to the assay mixtures in some cases. Also, due to the gelatinous nature of the "aggregate" enzyme preparations, it was difficult to ensure that a constant amount of the enzyme protein (0.5mg.) was added to each sample during assay. Thus corrections were made for this source of error by estimating for DNA or protein at the end of assay. When hyflo supercel was used to coprecipitate

the nucleic acids, protein determinations were carried out, but when carrier albumin was used this was not possible and corrections were made by estimating the DNA present at the end of assay.

Estimation of Ribonuclease Activity present in the "Ramuz" Enzyme Preparation

Ribonuclease activity of this enzyme preparation was assayed by adding a known amount of isotopically-labelled RNA to the mixtures used for polymerase assay and measuring the amount of labelled RNA remaining after incubation at 37° for various lengths of time. Purified rat-liver nuclear RNA pre-labelled with (^{14}C) adenine was prepared using the method described for sheep-thyroid nuclear RNA in Section II. About 10 $\mu\text{g.}$ of this preparation along with 100 $\mu\text{g.}$ yeast RNA as carrier were added to the assay mixtures and the samples incubated at 37° for various lengths of time up to 1 hr. The RNA was separated and the total counts in the RNA fraction measured as described above. The specific activity of the RNA was also determined as counts/min./mg. RNA.

RESULTS AND DISCUSSION

Studies using the Nuclear Enzyme Fraction prepared by the Method of Weiss (1960)

It was found that the DNA-dependent RNA polymerase activity of this enzyme preparation from sheep-thyroid nuclei varied considerably with different batches of nuclei. Fig. 26 shows the time course of incorporation of (^{32}P) UMP from ($\alpha\text{-}^{32}\text{P}$) UTP into RNA catalysed by enzyme preparations from sheep-thyroid and rat-liver nuclei in the presence or absence of 10% saturated $(\text{NH}_4)_2\text{SO}_4$. It can be seen that both enzyme preparations incorporate labelled UMP into RNA over the 40-min. incubation period, reaching an optimum after about 30 min. The lower values after 40-min. incubation are possibly due to nuclease activity present in the enzyme-DNA complexes. Table 18 shows some properties of the sheep-thyroid enzyme preparation. For its action, this enzyme requires the presence of all four ribonucleosides triphosphates and the reaction is inhibited by actinomycin D. The incorporation is known to be into RNA since the RNA fraction in each case was isolated for radioactivity measurements, and thus this

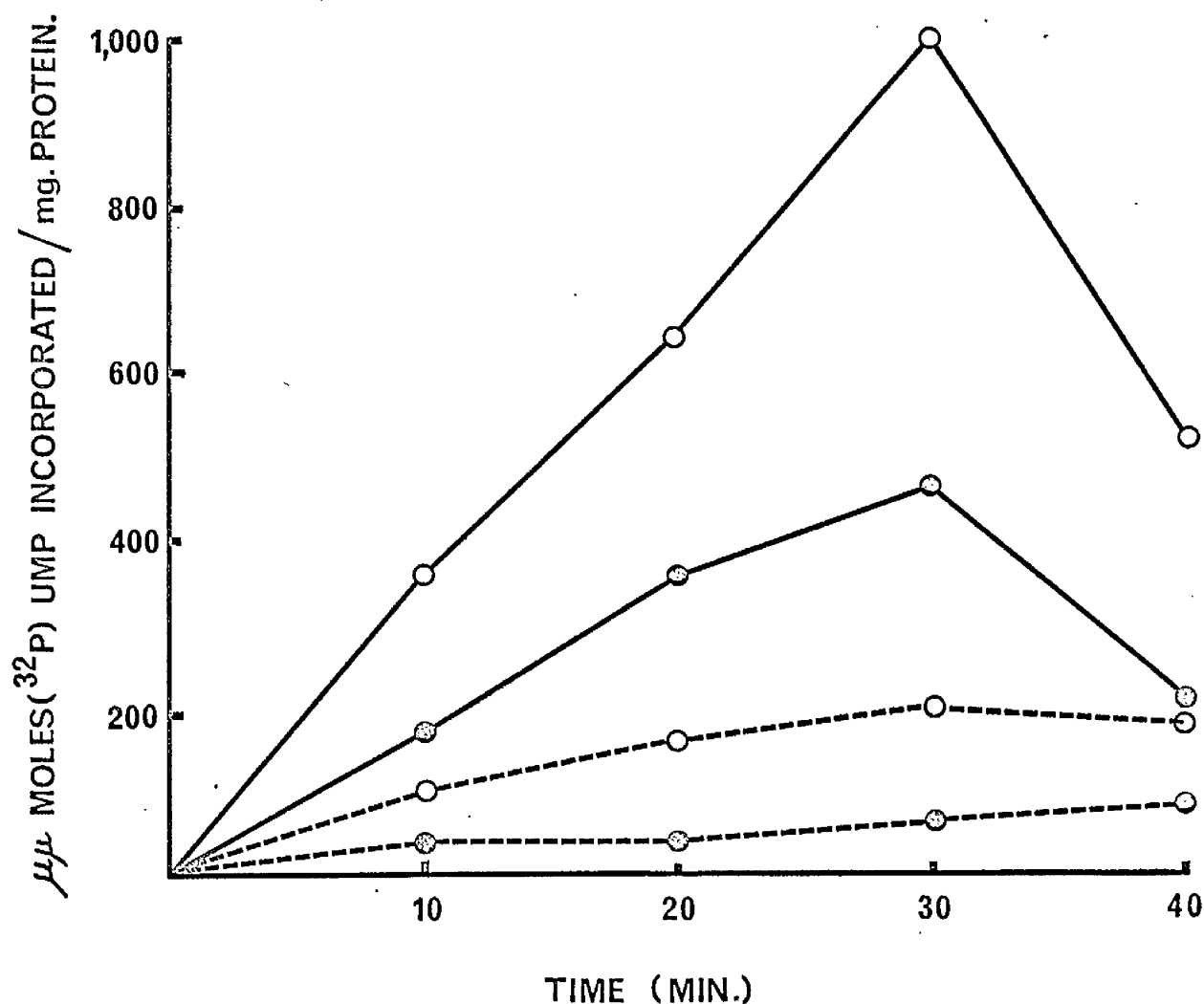


Fig. 26

Time course of incorporation of (^{32}P) UMP residues from $(\alpha\text{-}^{32}\text{P})$ UTP into RNA/mg. protein using "Weiss" enzyme preparations from rat-liver or sheep-thyroid nuclei in the presence or absence of 10% saturated $(\text{NH}_4)_2\text{SO}_4$. The unbroken lines represent the samples incubated with $(\text{NH}_4)_2\text{SO}_4$ and the broken lines those incubated without $(\text{NH}_4)_2\text{SO}_4$. •, sheep-thyroid enzyme; o, rat-liver enzyme. Each point is the mean result of three experiments.

Table 18Properties of the "Weiss" Enzyme Preparation from Sheep-Thyroid Nuclei

<u>Assay Conditions</u>		<u>μmoles (³²P) UMP incorporated into RNA/mg. Protein</u>
<u>Additions</u>	<u>Omissions</u>	
-	-	355
-	(NH ₄) ₂ SO ₄	45
-	ATP	27
-	CTP	33
-	ATP, CTP and GTP	30
-	(NH ₄) ₂ SO ₄ , ATP, CTP and GTP	19
actinomycin D (10μg.)	-	56
actinomycin D (10μg.)	(NH ₄) ₂ SO ₄	11

Samples were incubated for 20 min. at 37° with (α-³²P) UTP and the uptake of (³²P) UMP into RNA measured. The results are the mean values from two experiments performed in duplicate.

enzyme fraction from sheep-thyroid nuclei possesses DNA-dependent RNA polymerase activity. Both Fig. 26 and Table 18 show that the presence of 10% saturated $(\text{NH}_4)_2\text{SO}_4$ in the assay mixture affects the enzyme preparation resulting in increased synthesis of RNA. This effect has previously been reported for "Weiss" enzyme preparations from the nuclei of HeLa cells (Goldberg, 1961), chicken liver (Weill, Busch, Chambon and Mandel, 1963) and rat ventral prostate (Hancock, Jurkowitz and Jurkowitz, 1965). However the exact mechanism by which $(\text{NH}_4)_2\text{SO}_4$ causes stimulation is not yet known. It may be due to some alteration in the protein-DNA complex providing more DNA template for the polymerase enzyme and also to solubilization of the enzyme preparation.

Effect of TSH on the "Weiss" Enzyme Preparation from Sheep-Thyroid Nuclei

The TSH preparation used throughout the experiments described in this section was the Armour preparation (1 i.u./mg. protein) unless otherwise stated. TSH (0.05 i.u.) was added to the assay mixtures at the start of incubation to see

if it stimulated the DNA-dependent RNA polymerase activity of the enzyme preparation. However, the data shown in Table 19 demonstrate that TSH added directly to the assay medium had no stimulatory effect, and in fact appeared to have a slight inhibitory effect. This inhibition could perhaps be due to binding of the TSH protein to the DNA-enzyme complex during the reaction.

Sheep-thyroid nuclei were then preincubated for 15 min. at 37° in the presence or absence of TSH (0.1 i.u.), as described in Section II, prior to the preparation of the "Weiss" enzyme fractions. A preincubation time of 15 min. was chosen since the stimulatory effect of TSH on (¹⁴C)-adenine uptake into nuclear RNA was fully effective at this time under these experimental conditions (Fig. 20). The effect of addition of 0.4 μmoles of each of the four ribonucleoside triphosphates to the nuclear incubation mixture was also examined. The results obtained for assays of DNA-dependent RNA polymerase activity using enzyme preparations from control, TSH-treated and unincubated nuclei are shown in Table 20. It can be seen that treatment of sheep-thyroid nuclei under conditions where TSH is known to stimulate

Table 19

Effect of Addition to the Assay Medium of TSH on the Activity of the "Weiss" Enzyme Preparation from Sheep-Thyroid Nudei

<u>Time of Incubation</u> (min.)	<u>μmoles (32P UMP) incorporated into RNA/mg. Protein</u>	
	<u>Control</u> $-(\text{NH}_4)_2\text{SO}_4 + (\text{NH}_4)_2\text{SO}_4$	<u>TSH-treated</u> $-(\text{NH}_4)_2\text{SO}_4 + (\text{NH}_4)_2\text{SO}_4$
10	23	72
20	37	174
30	68	321
		62
		297

The uptake of (32 P) UMP residues from (32 P) UTP into RNA was measured in the presence or absence of TSH (0.05 i.u.). Each value is the mean result of three experiments performed in duplicate, all of which showed the same pattern of results.

Table 20

Effect of Preincubating Sheep-Thyroid Nuclei in the Presence or Absence of TSH on the Activity of the "Weiss" Enzyme Fractions Isolated after Incubation

<u>Time of Incubation (min.)</u>	<u>Addition of triphosphates during preincubation</u>	<u>μ moles (^{14}C) UMP incorporated into RNA/mg. Protein</u>		
		<u>Nuclei Incubated</u>	<u>TSH-treated</u>	<u>Nuclei Unincubated</u>
		<u>Control</u> $-(\text{NH}_4)_2\text{SO}_4 + (\text{NH}_4)_2\text{SO}_4$	$-(\text{NH}_4)_2\text{SO}_4 + (\text{NH}_4)_2\text{SO}_4$	$-(\text{NH}_4)_2\text{SO}_4 + (\text{NH}_4)_2\text{SO}_4$
10	-	34	22	-
20	-	52	46	74
20	+	59	51	-

Sheep-thyroid nuclei were preincubated for 15 min. at 37° in the presence or absence of TSH (0.1 i.u.), prior to isolation of the "Weiss" enzyme fractions from the control and TSH-treated nuclei. The effect of addition of 0.4 μ moles each of the four ribonucleoside triphosphates to the nuclear preincubation medium was also tested. The uptake of (^{14}C) UMP residues from (^{14}C) UTP into RNA by these enzyme preparations was then assayed. The results are the mean values from two experiments, each of which showed the same pattern of results.

adenine uptake into RNA had no stimulatory effect on the DNA-dependent RNA polymerase activities of the "Weiss" enzyme fractions prepared from these nuclei. Also, preincubation of sheep-thyroid nuclei prior to preparation of these enzyme fractions resulted in a loss of polymerase activity, and addition of nucleoside triphosphates during nuclear preincubation had no effect on the polymerase activity of the enzyme preparations.

Studies using the Nuclear Enzyme Fractions
prepared by the Method of Ramuz et al. (1965a)

During isolation of the "Weiss" enzyme fraction from sheep-thyroid nuclei, part of the whole nucleus is discarded (see Fig. 25) and it may be that some nuclear fraction is lost which is essential for TSH stimulation of RNA synthesis. Accordingly sheep-thyroid nuclei were fractionated by the procedure of Ramuz et al. (1965a), and assays of DNA-dependent RNA polymerase activity carried out on the "Ramuz" and "Soluble" fractions obtained (see Fig. 25). The effect of addition of 10% saturated $(\text{NH}_4)_2\text{SO}_4$ to the assay mixtures was again examined, since it has been

shown in several instances that hormone treatment appears to affect DNA-dependent RNA polymerase activity differently in the presence or absence of $(\text{NH}_4)_2\text{SO}_4$ (Gorski, 1964; Pegg and Korner, 1965; Ramuz, Doly, Chambon and Mandel, 1965b).

In agreement with results obtained by Ramuz et al. (1965a) for rat-liver nuclei, DNA-dependent RNA polymerase activity was found in both the "Ramuz" and "Soluble" fractions from sheep-thyroid nuclei. The "Soluble" fraction was found to contain only about 2% of the total nuclear DNA, and thus exogenous calf-thymus DNA had to be added to the assay mixtures in order to assay the DNA-dependent RNA polymerase activity of this fraction. The effect of pH variation on the polymerase activities of the "Soluble" and "Ramuz" enzyme fractions from sheep-thyroid nuclei was investigated. The results in Fig. 27 show that the pH optimum for the "Soluble" enzyme fraction is about 7.2, whereas that of the "Ramuz" enzyme fraction is about 8.4. The results also show that addition of $(\text{NH}_4)_2\text{SO}_4$ to the assay mixture stimulates the activity of the "Ramuz" enzyme but

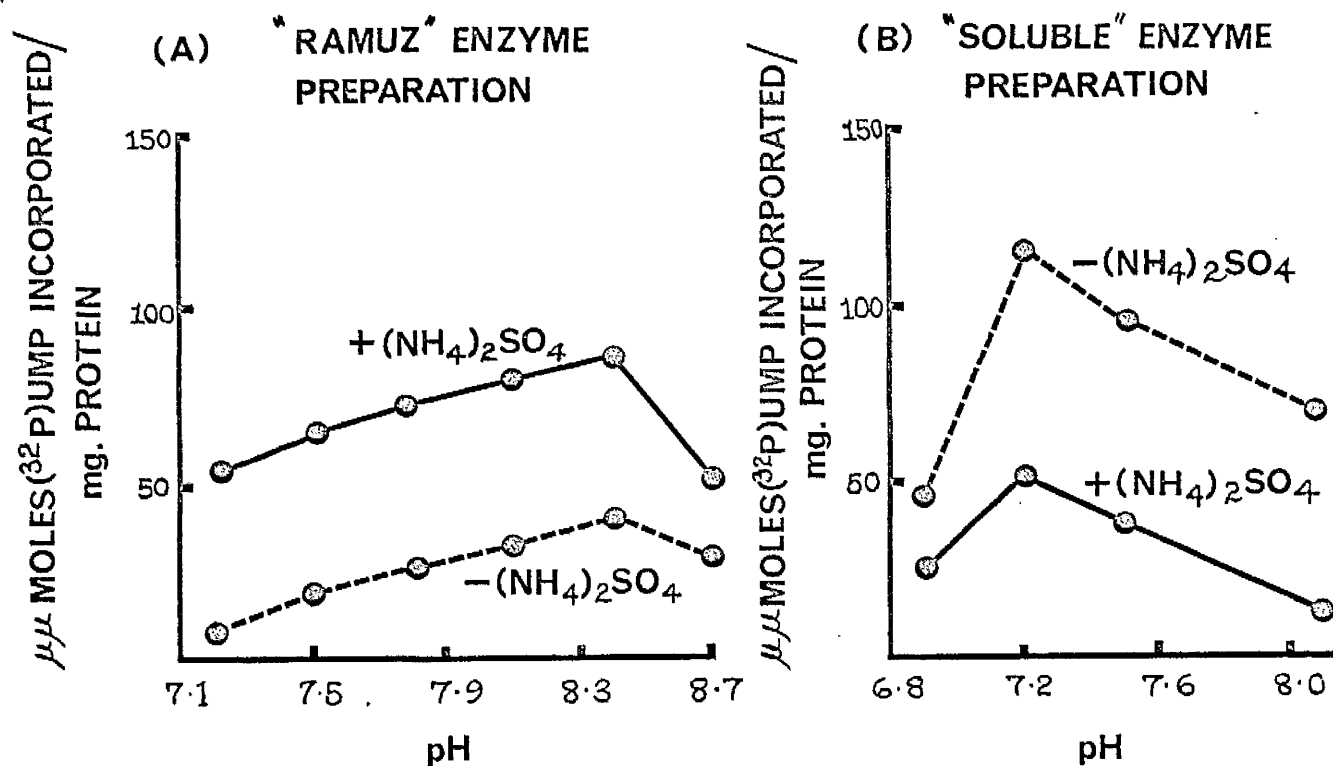


Fig. 27

Effect of pH variation on the uptake of (^{32}P) UMP residues of (α - ^{32}P) UTP into RNA/mg. protein by (a) the "Ramuz" enzyme preparation or (b) the "Soluble" enzyme preparation from sheep-thyroid nuclei incubated with 1.5 μmoles MnCl_2 for 20 min. at 37° in the presence or absence of 10% saturated $(\text{NH}_4)_2\text{SO}_4$ at the appropriate pH. Results are the mean values from two experiments.

inhibits that of the "Soluble" enzyme. This agrees with the findings reported for rat-liver nuclear enzyme fractions (Ramuz et al., 1965a). Further studies on these enzyme fractions from sheep-thyroid nuclei were then carried out.

Properties of the "Ramuz" Enzyme Preparation

Tables 21 and 22 show some properties of the "Ramuz" enzyme preparation. The uptake of UMP residues from UTP into RNA catalysed by this enzyme fraction was inhibited by omitting the other three ribonucleoside triphosphates from the assay medium or by the addition of actinomycin D or deoxyribonuclease at the start of assay. Also nucleotide residues from all four ribonucleoside triphosphates were incorporated to a similar extent, and the product of the reaction was ribonuclease-sensitive. Thus this enzyme fraction possesses DNA-dependent RNA polymerase activity. The effect of replacing Mn^{2+} by Mg^{2+} in the assay mixtures was also investigated. The amounts of $MnCl_2$ (1.5 μ moles) and $MgCl_2$ (5 μ moles) were chosen because they have been shown to be in the optimal region for the DNA-dependent RNA polymerase activity of rat-liver nuclei (Weiss, 1960;

Table 21

Properties of the "Rammuz" Enzyme Preparation from Sheep-Thyroid Nuclei - I

Additions or Omissions	Incubation Conditions	Labelled Nucleoside Triphosphate	μmoles Labelled Nucleotide incorporated into RNA/mg. Protein	
			$-(\text{NH}_4)_2\text{SO}_4$	$+(\text{NH}_4)_2\text{SO}_4$
Complete		(^3H) UTP	151	360
- ATP, CTP and GTP		"	9	34
+ actinomycin D (10μg.)		"	26	73
+ DNase (5μg.)		"	88	178
+ RNase (2μg.)*		"	13	54
- MnCl_2 ; + MgCl_2 (5μmoles)		"	155	192
Complete		(^3H) UTP	-	180
Complete		(^3H) ATP	-	234
Complete		(^3H) CTP	-	150
Complete		(^3H) GTP	-	231

The samples were incubated for 20 min. at 37° in the presence or absence of 10% saturated $(\text{NH}_4)_2\text{SO}_4$ with labelled nucleoside triphosphate and the incorporation of labelled nucleotide into RNA measured. The results are the mean values from two experiments.

* After 20-min. incubation period, samples were incubated for a further 10 min. at 37° with RNase (2μg./assay).

Table 22

Properties of the "Ramuz" Enzyme Preparation from Sheep-Thyroid Nuclei - II

<u>Effects studied</u>		<u>μmoles (³²P) UMP incorporated into RNA/mg. Protein</u>
Variation in Enzyme Concentration:		
0.2mg. protein per assay		41
0.5mg. " "		99
0.7mg. " "		205
Variation in MnCl ₂ Concentration:		
0.5μmoles per assay		73
1.5μmoles " "		93
2.5μmoles " "		61
*Addition of Energy-Generating System: (phosphocreatine/ creatine kinase)		
control		105
+ energy generator		113

Samples were incubated for 20 min. at 37° in the presence of 10% saturated (NH₄)₂SO₄ and the uptake of the (³²P) UMP residues from (α-³²P) UTP into RNA measured.

*Creatine phosphate (1.5mg.) and phosphocreatine kinase (10μg.) were added to the assay mixtures.

Widnell and Tata, 1964). Both Fig. 28 and Table 21 show that the activity of the "Ramuz" enzyme fraction was stimulated by addition of $(\text{NH}_4)_2\text{SO}_4$ to the assay mixture to a much greater extent when Mn^{2+} rather than Mg^{2+} was present as catalyst. Also the activity in the absence of $(\text{NH}_4)_2\text{SO}_4$ was slightly greater when Mg^{2+} was present. Table 22 shows that an amount of the enzyme fraction equivalent to 0.5mg. protein is satisfactory for general use in these polymerase assays and that an MnCl_2 concentration of 1.5 μ moles/assay also appears to be optimal. The addition of an energy-generating system appears to have little effect on enzyme activity suggesting that the levels of the nucleoside triphosphates, particularly ATP, used in these polymerase assays are not rate-limiting (Table 22).

Properties of the "Soluble" Enzyme Preparation

Table 23 shows some properties of the "Soluble" enzyme fraction from sheep-thyroid nuclei. The uptake of (^3H) UMP from (^3H) UTP into RNA requires an exogenous source of DNA, and is inhibited by omitting the other three ribonucleoside triphosphates from the assay medium.

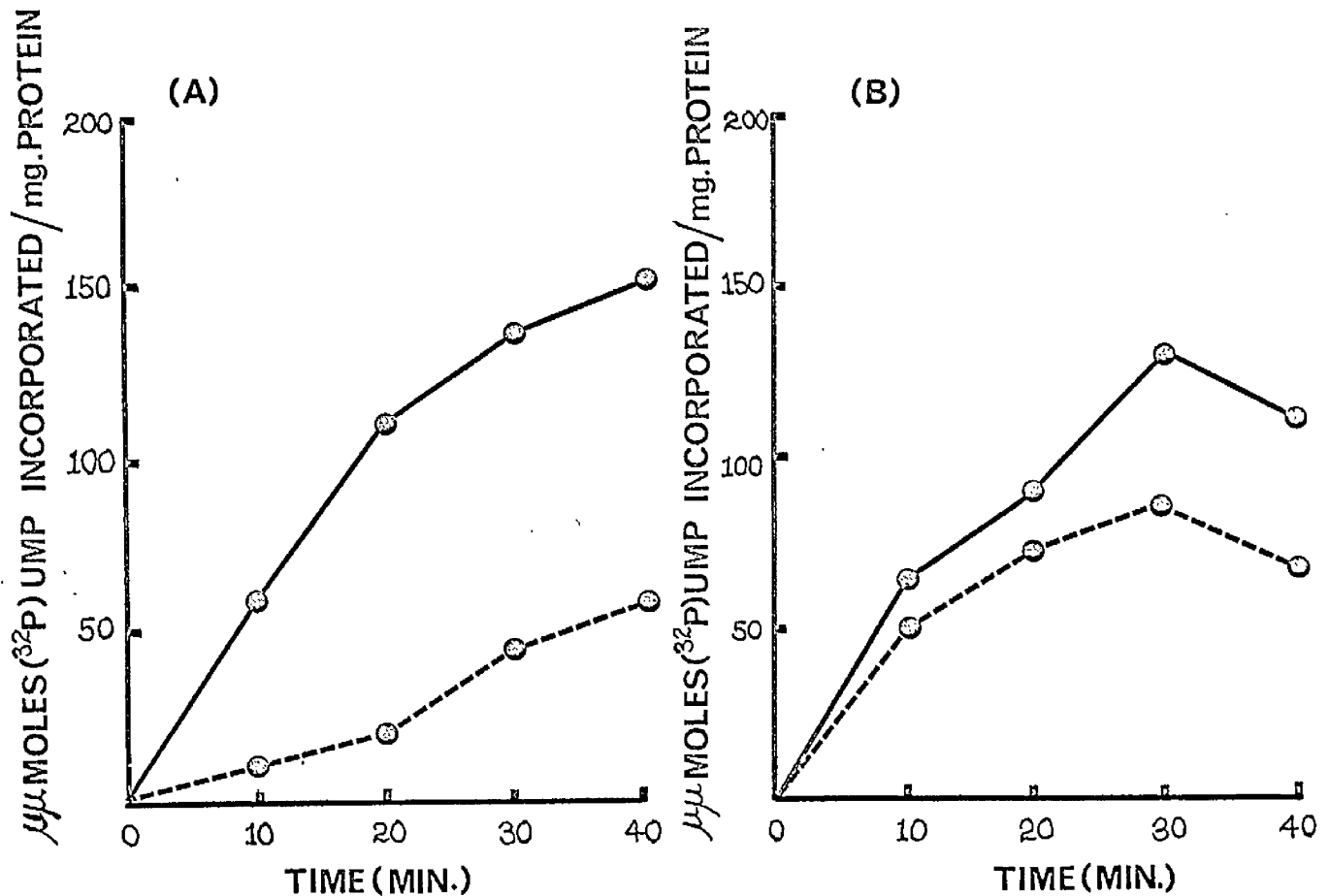


Fig. 28

Time course of incorporation of (^{32}P) UMP residues from $(\alpha\text{-}^{32}\text{P})$ UTP into RNA/mg. protein by the "Ramuz" enzyme preparation from sheep-thyroid nuclei (a) in the presence of $1.5 \mu\text{moles MnCl}_2$, or (b) in the presence of $5 \mu\text{moles MgCl}_2$. The unbroken lines represent the samples incubated in the presence of 10% saturated $(\text{NH}_4)_2\text{SO}_4$ (pH 8.4), and the broken lines those incubated in the absence of $(\text{NH}_4)_2\text{SO}_4$. Each point is the mean result from two experiments.

Table 23

Properties of the "Soluble" Enzyme Preparation from Sheep-Thyroid Nuclei

<u>Incubation Conditions</u>		<u>µmoles Labelled Nucleotide incorporated into RNA/mg. Protein</u>	
<u>Additions or Omissions</u>	<u>Labelled Nucleoside Triphosphate</u>		
Complete	(³ H) UTP	174	
+ (NH ₄) ₂ SO ₄	"	53	
- calf-thymus DNA	"	32	
- ATP, CTP and GTP	"	32	
+ actinomycin D (10µg.)	"	29	
+ DNase (5µg.)	"	5	
+ RNase (2µg.)*	"	57	

Complete	(³ H) UTP	116
Complete	(³ H) ATP	123
Complete	(³ H) CTP	94
Complete	(³ H) GTP	76

Samples were incubated for 20 min. at 37° in the absence of (NH₄)₂SO₄ and the incorporation of labelled nucleotide residues from a labelled nucleoside triphosphate into RNA measured.

The results are the mean values from two experiments.

*After 20-min. incubation period, samples were incubated for a further 10 min. at 37° with RNase (2µg./ assay).

Actinomycin D and DNase also inhibit the reaction, once more indicating DNA-dependence. The "Soluble" enzyme catalyses the incorporation of all four labelled ribonucleoside triphosphates into RNA to a similar extent, and the product of the reaction is ribonuclease-sensitive although not to such an extent as the product of the "Ramuz" enzyme preparation. The reason for this is not known, but the RNA product of the "Soluble" polymerase reaction will have a different base composition from that of the "Ramuz" polymerase reaction, since an exogenous calf-thymus DNA template was added to the former to assay its DNA-dependent RNA polymerase activity. Also the RNA product could be bound to the "Soluble" enzyme protein or to DNA in such a way that it is ribonuclease-resistant, or else the RNA formed contains nucleotide sequences, such as poly A, which are not degraded by pancreatic ribonuclease.

The polymerase reaction catalysed by the "Soluble" enzyme preparation, unlike the "Weiss" or "Ramuz" aggregate enzyme preparations, is inhibited by $(\text{NH}_4)_2\text{SO}_4$ (Fig. 27 and Table 23). To assay the DNA-dependent RNA polymerase

activity of the "Soluble" enzyme fraction, an exogenous DNA primer has to be added, whereas the DNA bound in the nucleoprotein of the aggregate enzyme preparation serves as template for the polymerase reaction and $(\text{NH}_4)_2\text{SO}_4$ may alter the structure of the latter so as make more DNA available for transcription by the polymerase enzyme.

Fig. 29 shows the time course of incorporation of (^3H) UMP residues from (^3H) UTP into RNA catalysed by the "Soluble" enzyme preparation. In the absence of $(\text{NH}_4)_2\text{SO}_4$, Mn^{2+} appears to be more effective as cofactor than Mg^{2+} under the conditions of assay employed.

Effect of TSH on the "Ramuz" and "Soluble" Enzyme Fractions from Sheep-Thyroid Nuclei

Sheep-thyroid nuclei were incubated for 15 min. at 37° in the presence or absence of TSH (0.1 i.u.), as described previously, and "Ramuz" and "Soluble" enzyme fractions prepared from control and TSH-treated nuclei. Assays of DNA-dependent RNA polymerase activity were then carried out over a 20-min. incubation period in the presence of Mn^{2+} ; the "Ramuz" enzyme fraction was assayed

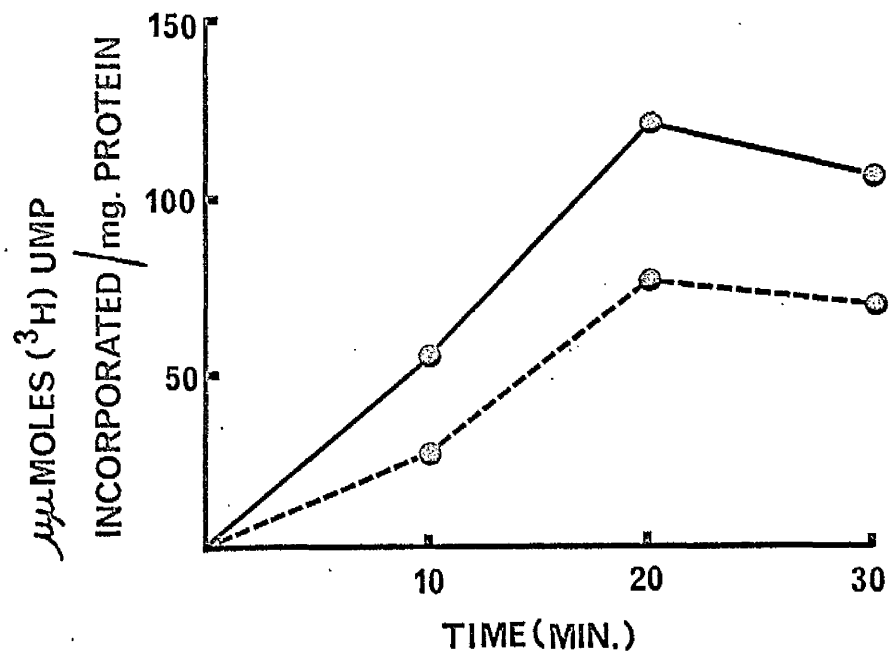


Fig. 29

Time course of incorporation of (³H) UMP residues from (³H) UTP into RNA/mg. protein in the absence of (NH₄)₂SO₄ using the "Soluble" enzyme preparation from sheep-thyroid nuclei. The unbroken line represents the samples incubated in the presence of 1.5 μmoles MnCl₂, and the broken line those incubated in the presence of 5 μmoles MgCl₂.

both in the presence and absence of the saturated $(\text{NH}_4)_2\text{SO}_4$ and the "Soluble" enzyme fraction only in the absence of $(\text{NH}_4)_2\text{SO}_4$. The results in Table 24 show that preincubation of sheep-thyroid nuclei with TSH prior to enzyme preparation resulted in an increase in the DNA-dependent RNA polymerase activity of the "Ramuz" enzyme preparation in the presence of $(\text{NH}_4)_2\text{SO}_4$, but there was no detectable increase in the absence of $(\text{NH}_4)_2\text{SO}_4$. The activity of the "Soluble" enzyme preparation, which was very low, appeared to be increased by a very small amount on treatment of the nuclei with TSH. However TSH (0.05 i.u.) added directly to the polymerase assay mixtures had no stimulatory effect on the activity of either the "Ramuz" or the "Soluble" enzyme preparations from control nuclei (Table 24). Again incubation of the nuclei prior to preparation of the enzyme fractions resulted in a loss of DNA-dependent RNA polymerase activity from both enzyme fractions, and the activity of the enzyme preparations as well as the degree of stimulation in activity of the "Ramuz" enzyme fraction caused by $(\text{NH}_4)_2\text{SO}_4$ varied considerably with preparations from different batches of nuclei.

Table 24

Effect of TSH Treatment on the Activities of the "Ramuiz" and "Soluble"
Enzyme Fractions from Sheep-Thyroid Nuclei

<u>Enzyme</u>	<u>Sample</u>	<u>μmoles (³H) UMP incorporated into RNA/mg. Protein</u> <u>- (NH₄)₂SO₄ + (NH₄)₂SO₄</u>
"Ramuiz"	Control	26
	TSH-treated during preincubation	20
	+ TSH (0.05 i.u.) added to polymerase assay	55
"Soluble"	Control	16
	TSH-treated during preincubation	20
	+ TSH (0.05 i.u.) added to polymerase assay	10

"Ramuiz" and "Soluble" enzyme fractions were prepared from sheep-thyroid nuclei which had been preincubated for 15 min. at 37° in the presence or absence of TSH (0.1 i.u.). Assays of DNA-dependent RNA polymerase activity of each fraction were then carried out. The samples were incubated for 20 min. at 37° in the presence of (³H) UTP and the incorporation of (³H) UMP residues into RNA measured. TSH (0.05 i.u.) was also added at the start of assay to some mixtures containing the control enzyme fraction. The results are the mean values from three experiments, all of which showed the same pattern of results.

Since apparent stimulation of the DNA-dependent RNA polymerase activity of the "Ramuz" enzyme fraction was obtained by incubating the nuclei for 15 min. with TSH before enzyme fractionation (Table 24), and since the "Weiss" enzyme preparation was not responsive under similar nuclear incubation conditions, (Table 20), it was decided to prepare both enzyme fractions from the same nuclei which had been preincubated in the absence and presence of TSH, and to assay DNA-dependent RNA polymerase activity in each case. The data shown in Table 25 are in agreement with previous results. Preincubation of the nuclei with TSH prior to preparation of the enzyme fractions did not result in a stimulation of activity of the "Weiss" enzyme preparation, whereas the activity of the "Ramuz" enzyme preparation assayed in the presence of $(\text{NH}_4)_2\text{SO}_4$ was again increased. When the enzyme activities are expressed per mg. DNA instead of per mg. protein, a similar pattern of results is obtained. These results suggest that some nuclear factor which is essential for TSH stimulation of polymerase activity is present in the "Ramuz" enzyme fraction and has been

Table 25

Comparison of the Effect of TSH Treatment on the Activities of the "Ramuz" and "Weiss" Enzyme Fractions from Sheep-Thyroid Nuclei

Enzyme	Sample	$\mu\text{moles } (^3\text{H}) \text{ UMP incorporated into RNA/mg. Protein}^*$	
		$-(\text{NH}_4)_2\text{SO}_4$	$+(\text{NH}_4)_2\text{SO}_4$
"Ramuz"	Control	49	75
	TSH-treated	46	126
"Weiss"	Control	26	114
	TSH-treated	24	107

"Ramuz" and "Weiss" enzyme fractions were prepared from sheep-thyroid nuclei which had been preincubated for 15 min. at 37° in the presence or absence of TSH (0.1 i.u.). Assays of DNA-dependent RNA polymerase activity of each enzyme fraction were then carried out. The samples were incubated for 20 min. at 37° in the presence of (^3H) UTP and the incorporation of (^3H) UMP residues into RNA measured. The results are the mean values from three experiments, all of which showed the same pattern of results.

*The data, when expressed per mg. DNA, gave a similar pattern of results.

discarded during preparation of the "Weiss" enzyme fraction at the stage of KCl treatment (see Fig. 25). Statistical analysis of the data from individual experiments, the means of which are shown in Table 24 and 25, demonstrates that treatment of sheep-thyroid nuclei with TSH resulted in a significant increase ($P < 0.05$) of 70% in the uptake of labelled UMP from UTP into RNA catalysed by the "Ramuz" enzyme fraction, when the polymerase assays were carried out in the presence of 10% saturated $(\text{NH}_4)_2\text{SO}_4$.

Many of the enzyme preparations isolated from sheep-thyroid nuclei were of very low activity and in later experiments the "Ramuz" and "Soluble" enzyme fractions were isolated from the nuclei using ice-cold 0.05M-tris-HCl (pH 7.4) containing 0.01M-2-mercaptoethanol to try and prevent inactivation of the polymerase enzyme. When the "Ramuz" and "Soluble" enzyme fractions were then prepared from sheep-thyroid nuclei which had been preincubated for 15 min. in the presence or absence of TSH, it was found that the polymerase activity of the "Ramuz" enzyme fraction isolated from the TSH-treated nuclei was greater in assays carried

out both in the presence and absence of $(\text{NH}_4)_2\text{SO}_4$ (Table 26). It can be seen also, that preincubation of the nuclei with more highly purified TSH (N.I.H.), in place of the Armour TSH, produced the same effects on the activity of the "Ramuz" enzyme preparations. However, when MgCl_2 (5 μ moles) replaced MnCl_2 (1.5 μ moles) in the polymerase assay mixtures, no stimulatory effect of TSH was obtained (Table 26). Also it appears that the activity of the "Soluble" enzyme fraction is not affected to any great extent on treatment of the nuclei with TSH, and no significant difference in activity of this fraction was obtained when Mn^{2+} was replaced by Mg^{2+} in the assay mixture.

Effect of Puromycin on the "Ramuz" and "Soluble"
Enzyme Fractions from Sheep-Thyroid Nuclei

The effect of puromycin on this system was then examined (Tables 27 and 28). Nuclei were preincubated for 15 min. at 37° with or without TSH (0.1 i.u.) and in the presence or absence of puromycin (100 μ g./ml.). "Ramuz" enzyme fractions were then prepared from nuclei treated under the various incubation conditions and assays of DNA-

Table 26

Effect of TSH on the Activities of the "Ramuz" and "Soluble" Enzyme Fractions prepared from Sheep-Thyroid Nuclei in the Presence of 2-Mercaptoethanol

Enzyme	Sample	$\mu\text{moles } (^3\text{H}) \text{ UMP incorporated into RNA/mg. Protein}$	
		$-(\text{NH}_4)_2\text{SO}_4$	$+(\text{NH}_4)_2\text{SO}_4$
"Ramuz"	Control	52	102
	TSH-treated	63	125
	TSH-treated (N.I.H.)	68	134
	Control; $-\text{MnCl}_2$, $+\text{MgCl}_2^*$	53	91
"	TSH-treated; $-\text{MnCl}_2$, $+\text{MgCl}_2^*$	43	93
"Soluble"	Control	13	-
	TSH-treated	14	-
	TSH-treated (N.I.H.)	17	-
	Control; $-\text{MnCl}_2$, $+\text{MgCl}_2^*$	16	-
"	TSH-treated; $-\text{MnCl}_2$, $+\text{MgCl}_2^*$	11	-

"Ramuz" and "Soluble" enzyme fractions were prepared from sheep-thyroid nuclei which had been pre-incubated for 15 min. at 37° in the presence or absence of TSH or TSH (N.I.H.) (0.1 i.u.). Assays of DNA-dependent RNA polymerase activity of each fraction were then carried out. Samples were incubated for 20 min. at 37° in the presence of (^3H) UTP and the incorporation of (^3H) UMP into RNA measured. The results are the mean values from two experiments, each showing the same pattern of results.

* MgCl_2 (5 μmoles) replaced MnCl_2 (1.5 μmoles) during assay of polymerase activity.

Table 27

Effects of TSH and Puromycin on the Activity of the "Ramuz" Enzyme Fraction prepared from Sheep-Thyroid Nuclei in the Presence of 2-Mercaptoethanol

Expt. No.	Nuclear Preincubation Conditions	μ moles (^{14}C) UMP incorporated into RNA/mg. Protein			
		$-(\text{NH}_4)_2\text{SO}_4$ Control	$-(\text{NH}_4)_2\text{SO}_4$ TSH-treated	$-(\text{NH}_4)_2\text{SO}_4$	$-(\text{NH}_4)_2\text{SO}_4$
1.	Control	92	147	95	167
	Puromycin-treated	87	110	91	131
2.	Control	34	100	34	103
	Puromycin-treated	31	64	30	65

In each experiment sheep-thyroid nuclei were preincubated for 15 min. at 37° with or without TSH (0.1 i.u.) in the presence or absence of puromycin (100 μ g./ml.). "Ramuz" enzyme fractions were then prepared from nuclei treated under various incubation conditions and assays of DNA-dependent RNA polymerase activities carried out. The samples were incubated for 20 min. at 37° and the incorporation of (^{14}C) UMP from (^{14}C) UTP into RNA measured.

Table 28

Effects of Puromycin and TSH on the Activities of the "Ramuz" and "Soluble" Enzyme Fractions Prepared from Sheep-Thyroid Nuclei in the Presence of 2-Mercaptoethanol

Enzyme	Nuclear Preincubation Conditions	μmoles (^{14}C) AMP incorporated into RNA/mg. Protein			
		Control	$-(\text{NH}_4)_2\text{SO}_4$	TSH-treated	$-(\text{NH}_4)_2\text{SO}_4$
"Ramuz"	Control	88	149	77	153
"	Puromycin added along with TSH	73	104	59	70
"	Nuclei preincubated with puromycin for 10 min. before TSH addition	40	82	33	75
"Soluble"	Control	37	-	42	-
"	Puromycin added along with TSH	20	-	19	-
"	Nuclei preincubated with puromycin for 10 min. before TSH addition	18	-	13	-

Puromycin (100μg./ml.) was added to the incubation medium containing sheep-thyroid nuclei either at the time of addition of TSH (N.I.H.) (0.1 i.u.) or 10 min. beforehand. The nuclei were then incubated for 15 min. at 37°, followed by preparation of the "Ramuz" and "Soluble" enzyme fractions from each nuclear sample. Assays of DNA-dependent RNA polymerase activity were then carried out by measuring the incorporation of (^{14}C) AMP from (^{14}C) ATP into RNA after incubation for 10 min. at 37°. The results are the mean values from two experiments performed in duplicate, each of which showed the same pattern of results.

dependent RNA polymerase activity carried out. The samples were incubated for 20 min. at 37° and the incorporation of (¹⁴C) UMP from (¹⁴C) UTP into RNA measured. The results in Table 27 show that puromycin treatment of sheep-thyroid nuclei prior to preparation of the "Ramuz" enzyme fraction results in a decrease in the polymerase activity of the enzyme preparations. In experiment 1, however, it appears that puromycin does not abolish the small increment in labelled UMP incorporation from UTP into RNA produced by TSH. In experiment 2, where there is no apparent TSH stimulation, the puromycin values from both control and TSH-treated samples are the same. Further investigations were therefore carried out on the effect of puromycin. Puromycin (100µg./ml.) was added to an incubation medium containing sheep-thyroid nuclei either at the time of addition of TSH (N.I.H.) (0.1 i.u.) or 10 min. beforehand. The nuclei were then preincubated for 15 min. at 37° as before, followed by preparation of the "Ramuz" and "Soluble" enzyme fractions from each nuclear sample. Assays of DNA-dependent RNA polymerase activity were then carried out by measuring in

this case the incorporation of (^{14}C) AMP from (^{14}C) ATP into RNA after incubation for 10 min. at 37° . The results obtained are shown in Table 28. Preincubation of sheep-thyroid nuclei with TSH (N.I.H.) does not appear to increase the uptake of (^{14}C) AMP from (^{14}C) ATP catalysed by either the "Ramuz" or "Soluble" enzyme preparations. Also the incorporation of labelled AMP appears to be somewhat greater than the incorporation of labelled UMP into RNA under similar conditions of incubation (see Tables 26 and 28). Puromycin added to sheep-thyroid nuclei along with TSH caused an inhibition of the polymerase activities of both the "Ramuz" and "Soluble" enzyme preparations, and preincubation of the nuclei with puromycin prior to the addition of TSH further reduced the activities of the polymerase preparations.

Protein Content and Protein/DNA Ratios of the Various Enzyme Preparations from Sheep-Thyroid Nuclei

Comparisons were also made of the protein content and the protein/DNA ratios of the various nuclear enzyme preparations. The data presented in Table 29 show that the "Weiss" enzyme preparation contains about 75% of the total

Table 29

Protein Content and Protein/DNA Ratios of the Various Polymerase
Enzyme Fractions Isolated from Sheep-Thyroid Nuclei

<u>Enzyme Fraction</u>	<u>% Total Nuclear Protein*</u>	<u>Protein/DNA*</u>
Whole nuclei	100	3.0 \pm 0.003
"Weiss"	75.3 \pm 0.36	2.25 \pm 0.018
"Rammuz"	90.2 \pm 0.50	2.46 \pm 0.030
"Rammuz" [†] (nuclei incubated before enzyme preparation)	85.2 \pm 0.29	2.30 \pm 0.019
"Soluble"	9.9 \pm 0.52	>50
"Soluble" [†] (nuclei incubated before enzyme preparation)	14.8 \pm 0.29	>50

* Mean values with standard errors

† Sheep-thyroid nuclei were incubated for 15 min. at 37° before preparation of the enzyme fraction.

nuclear protein and most of the nuclear DNA. The amount of protein occurring in this fraction was not significantly altered by preincubation of sheep-thyroid nuclei with TSH or ribonucleoside triphosphates prior to enzyme preparation. The "Ramuz" enzyme preparation contains almost all the nuclear DNA and about 90% of the total nuclear protein, the remainder occurring in the "Soluble" enzyme fraction. However, if sheep-thyroid nuclei are preincubated for 15 min. at 37° prior to preparation of these enzyme fractions, the amount of protein subsequently occurring in the "Ramuz" enzyme fraction is significantly decreased ($P < 0.05$) to 85% with a corresponding increase in the amount of protein in the "Soluble" enzyme fraction (Table 29). Addition of TSH or puromycin to the nuclear incubation medium did not significantly alter this distribution of nuclear protein in the "Ramuz" and "Soluble" enzyme fractions.

Studies on the Ribonuclease Activity present in the "Ramuz" Enzyme Fraction from Sheep-Thyroid Nuclei

It was decided to measure the ribonuclease activity present in the "Ramuz" enzyme preparation because it had

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been observed that the enzyme preparations from sheep-thyroid nuclei showed low levels of polymerase activity and the measured rate of RNA synthesis diminished with time (Figs. 26 and 28). Also, when nuclei were incubated prior to preparation of the enzyme fractions, the polymerase activity of these enzyme preparations was always lower than those of enzyme fractions prepared from nuclei which had not been preincubated. This could be due perhaps to activation of ribonuclease during the preincubation period.

"Ramuz" enzyme fractions were prepared from sheep-thyroid nuclei which had been incubated for 15 min. at 37° in the presence or absence of TSH (0.1 i.u.) and also from nuclei which had not been preincubated. Assays were then carried out for various lengths of time up to 1 hr. at 37° in the presence of 10% saturated $(\text{NH}_4)_2\text{SO}_4$, but with (^{14}C)-labelled rat-liver RNA (10 $\mu\text{g.}$) and yeast RNA (100 $\mu\text{g.}$) replacing the labelled nucleoside triphosphate. The total number of counts in the RNA fraction and the specific activity of the RNA were measured in each case. The results shown in Table 30 (a) demonstrate that all these "Ramuz" aggregate

Table 30

Measurement of the Ribonuclease Activity present in the
"Ramuz" Enzyme Fractions of Sheep-Thyroid Nuclei

(a)		Total Counts/min. in RNA/mg. Protein		
Time (min.)	Control	TSH-treated	Unincubated	Unincubated + TSH*
0	3,300	3,150	3,100	3,220
20	2,980	2,800	2,840	2,910
40	2,600	2,540	2,510	2,490
60	2,230	2,120	2,200	2,190

(b)		Counts/min. / mg. RNA		
Time (min.)	Control	TSH-treated	Unincubated	Unincubated + TSH*
0	27,400	25,300	26,900	27,100
20	23,800	22,700	23,100	22,900
40	21,200	19,900	20,000	19,700
60	18,750	17,600	17,800	18,200

Assays were carried out using various "Ramuz" enzyme preparations under the conditions employed for measurement of DNA-dependent RNA polymerase activity in the presence of 10% saturated $(\text{NH}_4)_2\text{SO}_4$. (^{14}C)-labelled rat-liver nuclear RNA (10 $\mu\text{g.}$) and yeast RNA (100 $\mu\text{g.}$) were added to the assay mixtures at the start of incubation and the amount of label and RNA remaining after incubation for various lengths of time measured. The results are the mean values from two experiments, each of which showed the same pattern of results.

* TSH (0.05 i.u.) added (with the RNA) at the start of incubation.

enzyme preparations possess ribonuclease activity, and that the extent of degradation of RNA to acid-soluble fragments is the same whether the nuclei have been preincubated or not prior to enzyme preparation. Also, addition of TSH either during nuclear preincubation or directly to the assay mixtures had no effect on ribonuclease activity. As shown in Table 30 (b), the specific activity of the RNA also falls throughout the incubation period showing that the labelled nuclear RNA is being degraded in preference to the added yeast RNA. This may reflect some specificity of the nuclease enzyme or enzymes for nuclear RNA or else the preparation of yeast RNA used is contaminated with some inhibitors of ribonuclease activity. Assays of DNA-dependent RNA polymerase activity using the "Ramuz" enzyme preparation were usually carried out for a 20-min. incubation period, and from the results in Table 30 it can be seen that RNA is being degraded to acid-soluble fragments during this time interval. Thus the results of DNA-dependent RNA polymerase activity described earlier actually reflect both the DNA-dependent synthesis of and also the breakdown of RNA during assay. Preliminary studies were carried out in which bentonite, at a

final concentration of 0.5mg. %, was added during assays of polymerase activity to try and inhibit ribonuclease activity. However, the results obtained were not very satisfactory since bentonite absorbs ribonucleoside triphosphates and this resulted in poor duplication of results.

Comments on the Results Obtained from Assays
of DNA-Dependent RNA Polymerase Activity
using Various Fractions prepared from
Sheep-Thyroid Nuclei

Properties of the Enzyme Preparations

Using the method of Weiss (1960) an enzyme fraction which possessed DNA-dependent RNA polymerase activity was prepared from purified sheep-thyroid nuclei. This enzyme fraction, which contained most of the nuclear DNA and about 75% of the total nuclear protein (Table 29), behaved similarly to that isolated from rat-liver nuclei, except that the DNA-dependent RNA polymerase activity was lower (Fig. 26). This may be due to the time-lag during the removal of thyroid glands from the sheep and preparation of the nuclei, but may also reflect the possibly lower metabolic activity of sheep-thyroid cells as opposed to that of rat-liver cells. The

activity of this enzyme preparation was increased by the addition of 10% saturated $(\text{NH}_4)_2\text{SO}_4$ during assay (Fig. 26 and Table 18).

Sheep-thyroid nuclei were also fractionated into an aggregate and a soluble enzyme fraction by the method of Ramuz et al. (1965a). The "Ramuz" aggregate enzyme fraction contained almost all the nuclear DNA and about 90% of the total nuclear protein, the remainder occurring in the "Soluble" fraction (Table 29). Preincubation of sheep-thyroid nuclei prior to enzyme preparation however caused a significant decrease in the amount of protein subsequently found in the "Ramuz" enzyme fraction with a corresponding increase in the amount in the "Soluble" enzyme fraction (Table 29). In agreement with results obtained by Ramuz et al. (1965a) for rat-liver nuclei, both enzyme fractions from sheep-thyroid nuclei possessed DNA-dependent RNA polymerase activity (Tables 21 and 23). The studies on pH variation carried out indicate that the "Ramuz" aggregate enzyme fraction has a pH optimum about 8.4, whereas that of the "Soluble" enzyme fraction is about 7.2 (Fig. 27). This may reflect differences

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in the chemical composition of the two fractions. In the "Ramuz" enzyme fraction the polymerase enzyme is closely associated with DNA and histones, whereas the "Soluble" enzyme fraction contains less than 2% of the total nuclear DNA, and thus exogenous DNA has to be added to assay the DNA-dependent RNA polymerase activity of this enzyme fraction. Also, in the presence of 10% saturated $(\text{NH}_4)_2\text{SO}_4$, the polymerase activity of the "Ramuz" enzyme fraction like that of the "Weiss" enzyme fraction is increased, whereas that of the "Soluble" enzyme fraction is decreased (Fig. 27 and Tables 21 and 23). According to Chambon, Ramuz and Doly (1965), the stimulatory effect of $(\text{NH}_4)_2\text{SO}_4$ is the result of two phenomena which have opposite effects.

$(\text{NH}_4)_2\text{SO}_4$ is thought to decrease the linkages between histones and DNA thus facilitating the transcription of more DNA segments by the polymerase enzyme. Some evidence for this has come from studies of the base composition of the RNA formed by nuclei or nuclear fractions in vitro both in the presence and absence of $(\text{NH}_4)_2\text{SO}_4$. The RNA formed in the presence of $(\text{NH}_4)_2\text{SO}_4$ has a base composition more like

that of native DNA than that formed in the absence of $(\text{NH}_4)_2\text{SO}_4$ (Widnell, 1965; Chambon et al., 1965; Liao, Hnilica and Hurlbert, 1965). On the other hand, $(\text{NH}_4)_2\text{SO}_4$ also appears to inhibit the action of RNA polymerase enzyme complexed to DNA and to newly synthesized RNA, possibly as a result of the increase in ionic strength of the medium. The latter action of $(\text{NH}_4)_2\text{SO}_4$ apparently causes the inhibition of the "Soluble" enzyme preparation. Whether these two enzyme fractions contain the same polymerase enzyme or enzymes is not known, but Chambon et al. (1965) have suggested from studies on rat-liver that the same enzyme or enzymes are present in both fractions.

Widnell and Tata (1964) and Widnell (1965) have described two DNA-dependent RNA polymerase reactions in rat-liver nuclei. One reaction, the product of which is similar to ribosomal RNA, is activated by Mg^{2+} whereas the second reaction, activated by Mn^{2+} in the presence of $(\text{NH}_4)_2\text{SO}_4$, yields a product which is more DNA-like. Similar results were also obtained from studies of DNA-dependent RNA polymerase activity using uterine nuclei from ovariectomized

rats (Hamilton, Widnell and Tata, 1965). However, there is no proof as yet that the two reactions are due to two distinct polymerase activities since, in the presence of $(\text{NH}_4)_2\text{SO}_4$ as described above, the RNA product of the reaction would be expected to have a base composition more like DNA and different from that synthesized in the absence of $(\text{NH}_4)_2\text{SO}_4$. The time course studies carried out on the "Ramuz" enzyme preparation from sheep-thyroid nuclei (Fig. 28) are in agreement with the results of Widnell and Tata (1964).

$(\text{NH}_4)_2\text{SO}_4$ was found to have a greater stimulatory effect when Mn^{2+} rather than Mg^{2+} is used as catalyst, and, in the absence of $(\text{NH}_4)_2\text{SO}_4$, the Mg^{2+} -catalysed reaction was slightly greater than the Mn^{2+} -catalysed reaction. With the "Soluble" enzyme preparation, however, in the absence of $(\text{NH}_4)_2\text{SO}_4$ Mn^{2+} appears to be more effective as catalyst (Fig. 29). At present it is difficult to relate these findings to those of Tata and Widnell (1964) as evidence for two distinct polymerase activities. In the presence of $(\text{NH}_4)_2\text{SO}_4$, Mn^{2+} appears to cause more transcription of DNA by the "Ramuz" enzyme fraction than does Mg^{2+} whereas in the absence of $(\text{NH}_4)_2\text{SO}_4$, Mg^{2+} appears

to be slightly more effective, when used at these particular concentrations during assay. The variations in polymerase activity could also be due to different effects of these ions on the ribonuclease activity of the enzyme preparation, since it has been shown that the "Ramuz" enzyme preparation possesses considerable ribonuclease activity (Table 30).

Effect of TSH on the DNA-Dependent RNA Polymerase Activities of the Enzyme Preparations

Direct addition of TSH to the polymerase assay mixtures at the start of assay did not increase the DNA-dependent RNA polymerase activity of the "Weiss" or "Ramuz" aggregate enzyme fractions or the "Soluble" enzyme fraction (Tables 19 and 24). Preincubation of sheep-thyroid nuclei with TSH under conditions where it is known to stimulate the uptake of (^{14}C) adenine into nuclear RNA (Fig. 20) had no stimulatory effect on the DNA-dependent RNA polymerase activity of the "Weiss" enzyme preparation (Tables 20 and 25). However, when the "Ramuz" enzyme fraction was prepared from the TSH-treated nuclei, it was found that there was a significant increase ($P < 0.05$) in the incorporation of (^3H) UMP

residues from (^3H) UTP into RNA catalysed by this enzyme fraction when the polymerase assays were carried out in the presence of 10% saturated $(\text{NH}_4)_2\text{SO}_4$ and Mn^{2+} (Tables 24 and 25). However, when this enzyme fraction was prepared from TSH-treated nuclei in the presence of 2-mercaptoethanol to preserve enzyme activity, the uptake of labelled UMP from UTP into RNA was increased both in the presence and absence of $(\text{NH}_4)_2\text{SO}_4$ (Table 26). Also when more highly purified TSH (N.I.H.) was used, a similar effect was obtained (Table 26). No TSH-stimulation of uptake into RNA catalysed by the "Ramuz" enzyme fraction was however observed when MgCl_2 (5 μmoles) replaced MnCl_2 (1.5 μmoles) in the polymerase assay mixture (Table 26). This finding suggests that either Mn^{2+} and Mg^{2+} are catalysing two different enzyme reactions or that the action of TSH may possibly be similar to that of $(\text{NH}_4)_2\text{SO}_4$. The polymerase activity of the "Soluble" enzyme fraction, which was very low, appeared to be increased slightly on treatment of the nuclei with TSH, but the effect was not significant (Tables 24 and 26).

Since preincubation of sheep-thyroid nuclei with TSH

prior to preparation of the enzyme fractions did not increase the incorporation of labelled UMP from (^3H) UTP into RNA catalysed by the "Weiss" enzyme preparation but did enhance that catalysed by the "Ramuz" enzyme fraction (Table 25), this suggests that some nuclear factor, which is essential for this stimulatory action of TSH, is discarded during preparation of the "Weiss" enzyme fraction at the stage of KCl treatment (Fig. 25). However, it was found that the incorporation of (^{14}C) AMP residues from (^{14}C) ATP into RNA by the "Ramuz" enzyme fraction was not increased on preincubation of the nuclei with TSH prior to enzyme preparation, although under similar assay conditions, as mentioned above, the uptake of (^3H) UMP from (^3H) UTP was increased (Tables 26 and 28). The reason for this is not known, but the enzyme preparation used is very crude and may, in addition to catalysing DNA-dependent synthesis of RNA, catalyse homopolymer formation or extension of existing RNA strands by formation of poly A. This could result in production of poly A to such an extent as to mask the TSH effect on DNA-dependent RNA formation. This may

be the case, since comparing the data in Tables 26 and 28, the incorporation of labelled AMP residues from ATP was greater than the incorporation of labelled UMP residues from UTP into RNA under similar conditions of assay. Also there is evidence, in mammalian nuclei, of enzymes which from poly A. A DNA-dependent poly A-synthesizing enzyme from hen liver has been described by Chambon, Weill and Mandel (1963), and Edmonds and Abrams (1963) have described synthesis in calf-thymus nuclei of poly A from ATP by an ATP polymerase which depends on a polynucleotide present in the enzyme preparation.

Effect of Puromycin on the DNA-dependent RNA Polymerase Activities of the "Ramuz" and "Soluble" Enzyme Fractions

When puromycin (100 μ g.) was added during preincubation of sheep-thyroid nuclei, in the presence or absence of TSH, the DNA-dependent RNA polymerase activities of the "Ramuz" and "Soluble" enzyme fractions prepared from the nuclei after incubation were decreased. Both the incorporation of labelled UMP and AMP residues from UTP and ATP into RNA were affected (Tables 27 and 28). Table 28 also shows

that preincubation of the nuclei with puromycin for 10 min. prior to TSH addition further reduces the activities of the enzyme fractions isolated after nuclear incubation.

However, from these preliminary investigations, it is impossible to say whether or not the increment in incorporation of labelled UMP from UTP into RNA caused by preincubation of the nuclei with TSH is specifically inhibited by puromycin treatment but the data in Table 27 suggest that this is not the case. The fact that polymerase activity is decreased suggests that puromycin inhibits the synthesis of some nuclear protein, perhaps the polymerase enzyme, which has a rapid turnover, and which affects DNA-dependent RNA synthesis. The protein involved must constitute a very small proportion of the total nuclear protein, since there were no detectable changes in the protein composition of the enzyme fractions upon puromycin treatment of sheep-thyroid nuclei prior to enzyme isolation. In this connection, the puromycin studies described in Section II show that synthesis of some nuclear protein is also essential for the stimulatory action of TSH on adenine uptake into RNA (Tables 16 and 17) although no change in (^{14}C) leucine uptake

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into total nuclear protein could be detected under similar conditions of TSH treatment (Fig. 22). Studies carried out by Holland, (1963) and Summers, Noteboom and Mueller (1966) on the effect of puromycin on the nuclear DNA-dependent RNA polymerase activity of HeLa cells have shown that, after treatment of the intact cells with puromycin, there was a fall in DNA-dependent RNA polymerase activity as assayed in vitro using both intact nuclei and a "Weiss" enzyme fraction as source of polymerase and DNA. However, they suggest that the effect of puromycin is not directly on the synthesis of polymerase but on some nuclear protein concerned with ribosomal RNA synthesis.

The present studies demonstrate that sheep-thyroid nuclei possess an enzyme or enzymes which catalyse the DNA-dependent synthesis of RNA, and show some properties of the enzyme fractions which possess this enzyme activity. However the preliminary investigations carried out on the effect of TSH treatment of nuclei on the polymerase activity of the enzyme fractions prepared after nuclear incubation gave results which are difficult to interpret and further studies are necessary to

clarify the situation. In order to study the action of a hormone on a mammalian DNA-dependent RNA polymerase system, it would be necessary to employ either a chromatin fraction prepared in such a way as to prevent the gross aggregation obtained with the preparations described above or a purified system. A hormone action on DNA-dependent RNA polymerase activity, if indeed such a mechanism exists, can be mediated via several routes, such as the rate of synthesis or degradation of the polymerase enzyme, the amount of DNA active in transcription or allosteric effects in the enzyme protein itself producing conformational changes with an increase in activity. Thus it is necessary to be able to distinguish between actual polymerase activity, template ability of the DNA and the amount of enzyme present, before a hormone action on DNA-dependent RNA synthesis can be described in detail.

SUMMARY

1. Purified sheep-thyroid nuclei were fractionated either by the procedure of Weiss (1960) or by that of Ramuz et al. (1965a). The protein and DNA content of the "Weiss" and "Ramuz" aggregate enzyme fractions and the "Soluble" enzyme fraction were

determined after preparation from nuclei which in some cases had been treated under various conditions of incubation. It was found that preincubation of the nuclei prior to fractionation caused a significant decrease in the amount of protein occurring in the "Ramuz" enzyme fraction with a corresponding increase in amount in the "Soluble" enzyme fraction.

2. All these three enzyme fractions were found to possess DNA-dependent RNA polymerase activity. The DNA functioning as template for the polymerase reaction formed part of the "Weiss" and "Ramuz" enzyme fractions, whereas the "Soluble" enzyme fraction contained less than 2% of the total nuclear DNA, and thus exogenous DNA had to be added to assay the polymerase activity of this enzyme fraction.

3. When assays of DNA-dependent RNA polymerase activity were carried out in the presence of 10% saturated $(\text{NH}_4)_2\text{SO}_4$, the amount of RNA synthesized by the "Weiss" and "Ramuz" enzyme fractions was increased, whereas the amount synthesized by the "Soluble" enzyme fraction was decreased.

4. The pH optimum of the "Ramuz" enzyme fraction was about

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8.4, whereas that of the "Soluble" enzyme fraction was about

7.2. The DNA-dependent RNA polymerase activity of the

"Ramuz" enzyme fraction was not increased by the addition of an energy-generating system to the assay medium.

5. The increase in the amount of RNA synthesized by the "Ramuz" enzyme fraction due to the presence of 10% saturated $(\text{NH}_4)_2\text{SO}_4$ in the assay medium was found to be greater in the presence of MnCl_2 (1.5 μmoles) than MgCl_2 (5 μmoles), and in the absence of $(\text{NH}_4)_2\text{SO}_4$, Mg^{2+} was slightly more effective as catalyst than Mn^{2+} . The DNA-dependent RNA polymerase activity of the "Soluble" enzyme fraction assayed in the absence of $(\text{NH}_4)_2\text{SO}_4$ was found to be greater in the presence of Mn^{2+} than Mg^{2+} .

6. The "Ramuz" enzyme fraction was found to contain a ribonuclease enzyme or enzymes which degraded RNA to acid-soluble fragments under the incubation conditions used for assay of DNA-dependent RNA polymerase activity.

7. TSH added directly to the polymerase assay mixture did not increase the DNA-dependent RNA polymerase activities of the nuclear enzyme fractions. Preincubation of sheep-thyroid

nuclei with TSH for 15 min. prior to preparation of the enzyme fractions did not affect the DNA-dependent RNA polymerase activity of the "Weiss" enzyme fraction. However there was an increase in the incorporation of (^3H) UMP from (^3H) UTP into RNA catalysed by the "Ramuz" enzyme fraction when assayed in the presence of 10% saturated $(\text{NH}_4)_2\text{SO}_4$. When the "Ramuz" enzyme fraction was prepared from TSH-treated nuclei in the presence of 2-mercaptoethanol, the increase was apparent during polymerase assay both in the presence and in the absence of $(\text{NH}_4)_2\text{SO}_4$. However, when labelled ATP replaced labelled UTP in the polymerase assay mixtures, TSH treatment of the nuclei prior to enzyme preparation did not enhance the incorporation of labelled AMP from ATP into RNA catalysed by the "Ramuz" enzyme preparation. Also, when MgCl_2 (5 μmoles) replaced MnCl_2 (1.5 μmoles) in the polymerase assay mixtures, no increase in labelled nucleotide incorporation into RNA catalysed by the "Ramuz" enzyme preparation from TSH-treated nuclei was apparent.

8. Puromycin treatment of sheep-thyroid nuclei in the

presence or absence of TSH, prior to enzyme preparation, was found to decrease the DNA-dependent RNA polymerase activities of the "Ramuz" and "Soluble" enzyme preparations.

GENERAL DISCUSSION

Effect of Body Size on the Nucleic Acid and
Protein Content of the Thyroid Gland

The studies, described in Section I, on the nucleic acid and protein composition of the thyroid glands of different mammals demonstrate that the amount of protein in the thyroids of these mammals is maintained at a constant proportion of body weight, whereas the amounts of RNA and, to a lesser extent, of DNA are decreased with increasing body weight. Comparison of these results with histological studies on the dimensions of thyroid cells and of colloid vesicles (Teissier, 1939; Yagizawa, 1956) indicates that the larger mammals maintain a constant relationship between the amount of protein in the thyroid gland and body size by an increase in extracellular storage of protein (thyroglobulin) in the colloid vesicles. It was also found that the amount of RNA per cell diminished with increasing body size, and since RNA is intimately involved in the protein synthetic mechanism this suggests that the synthesis of thyroid protein, including thyroglobulin, may decline with increasing body size. The rate of degradation of thyroglobulin and of release of thyroid hormone from the gland are also probably diminished with

increasing body size since the extent of storage of thyroid protein in the colloid vesicles is increased in the larger mammals. Thus the turnover of thyroglobulin is likely to be slower in larger than in smaller species of mammal. Whether this reflects some intrinsic property of the thyroid cells themselves or is the result of decreased levels of circulating TSH caused by alterations in the metabolism of the anterior pituitary is not known at present. However, the mass of the pituitary gland varies as the 0.762 power of body weight (Brody, 1945) and this suggests that the rate of production and of release of TSH from the pituitary may also decline with increasing body size.

Effects of TSH on the Nucleic Acid and
Protein Metabolism of the Thyroid Gland

(a) Experiments using Thyroid Slices

Using calf-thyroid slices, Hall (1963) showed that TSH increases the uptake of labelled precursors into the purine bases of tissue RNA over a 3-hr. incubation period. He also demonstrated that addition of glucose or purine precursors to the incubation medium enhanced the uptake of (^{14}C) formate into RNA, both in the presence and absence of TSH, and indicated that the

effects of TSH and purine precursors on thyroid purine nucleotide synthesis are additive. TSH and glucose each stimulate the uptake of (^{14}C) adenine into calf-thyroid RNA in vitro, and additive effects are again obtained when both TSH and adenine are present (Hall and Tubmen, 1965). As mentioned previously, TSH also causes an increased oxidation of glucose by the hexose monophosphate pathway within as little as 5 min. after addition to thyroid slices in vitro (Field, Pastan, Johnson and Herring, 1960; Dumont, 1961). On the basis of these observations, Hall (1963) has proposed that the action of TSH on purine ribonucleotide and RNA synthesis is mediated by TSH stimulation of glucose oxidation by the hexose monophosphate pathway, thereby increasing the supply of available ribose for purine ribonucleotide synthesis.

Amounts of TSH in the physiological range are effective in stimulating uptake of purine precursors into thyroid RNA and the effect seems to be specific since the hormone had no stimulatory effect on purine and RNA synthesis in calf-liver or kidney slices (Hall, 1963). Also, other pituitary hormones, ACTH, FSH and growth hormone as well as cortisol and

3-5-3'-triiodothyronine added to the incubation caused no stimulation of (^{14}C) formate into RNA (Hall and Tubmen, 1965). Thus stimulation of purine ribonucleotide and RNA synthesis appears to be a specific action of TSH on the thyroid gland in vitro.

In agreement with the work of Hall, the studies described in Section II, using sheep-thyroid slices show that addition of TSH to the incubation medium stimulated the uptake of (^{14}C) adenine into thyroid RNA over a 3-hr. period (Fig. 16). This increase in (^{14}C) adenine uptake is first apparent in the nuclear RNA fraction of the thyroid slices, and is followed later by increased labelling of cytoplasmic RNA (Fig. 17). However TSH does not appear to have a detectable stimulatory effect on (^{14}C) adenine uptake into thyroid RNA during the first hr. of incubation, although the increase in RNA labelling due to TSH after 2 or 3 hr. incubation is fairly large. Sucrose density gradient analysis of RNA prepared from sheep-thyroid slices which had been incubated for 3 hr. in the presence or absence of TSH shows that TSH apparently increases uptake of (^{14}C) adenine into all RNA species (Fig. 18). This suggests that

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TSH is having a general effect on thyroid RNA synthesis at this time. Gorski and Nelson (1965) have obtained similar results for the effect of oestradiol-17 β on the labelling of RNA isolated from rat uterus 1 hr. after in vivo hormone administration. Also it has been shown that, shortly after in vivo administration of cortisol to adrenalectomized rats, synthesis of all three species of rat-liver RNA, ribosomal, transfer and DNA-like is stimulated by the steroid hormone (Wicks, Greenman and Kenney, 1965; Greenman, Wicks and Kenney, 1965). However, while the response of RNA is general involving each of the three major types of RNA, it cannot be concluded in these cases that the hormone effect is completely indiscriminate since the method of analysis employed, i.e. separation of RNA on sucrose density gradients, is not sensitive enough to detect small changes in specific RNA fractions.

Both puromycin and actinomycin D added at the start of incubation were found to inhibit the stimulatory action of TSH on (^{14}C) adenine uptake into thyroid slice RNA (Tables 11 and 12). However, when either of these inhibitors was added after 1 or 2 hr. incubation, the stimulatory effect of TSH on

(^{14}C) adenine uptake into RNA was partially restored suggesting that the synthesis of both RNA and protein during the first hr. of incubation is necessary for TSH action on thyroid RNA metabolism. Raghupathy, Abraham, Kerkof and Chaikoff (1964) failed to show a stimulatory effect of TSH on the uptake of (^{14}C) leucine into the protein of sheep-thyroid slices in vitro, and thus it appears that TSH does not affect general thyroid protein synthesis under these incubation conditions. However, if the protein necessary for the stimulatory action of TSH on RNA synthesis only contributes a small extent to total thyroid protein, the increased leucine uptake into this protein due to TSH would not be detected if synthesis of most thyroid protein, e.g. thyroglobulin, were unaffected by TSH administration during the period of incubation.

(b) Experiments using Isolated Nuclei

Since the stimulatory action of TSH was more extensive and occurred at an earlier time in the case of nuclear RNA (Fig. 17), the effect of TSH on the uptake of (^{14}C) adenine by isolated sheep-thyroid nuclei was then examined. It was found that the uptake of labelled adenine by sheep-thyroid nuclei was

stimulated by TSH, the magnitude of stimulation being roughly proportional to the logarithm of the amount of TSH added (Fig. 20). This effect of TSH, which was demonstrable within 20 min. of TSH addition to the nuclei, appears to be fairly specific since rat-liver nuclei were not responsive under similar conditions of incubation (Fig. 21), and addition of protein (bovine serum albumin) in place of TSH protein to the incubation medium had no effect on adenine uptake into sheep-thyroid nuclei (Table 13). Treatment of the nuclei with actinomycin D was found to inhibit the action of TSH on adenine uptake, indicating that this process involves DNA-dependent synthesis of RNA (Tables 16 and 17).

The studies of Hall (1963) and Hall and Tubmen (1965) described above demonstrate that TSH increases the uptake of purine precursors into RNA purines in vitro, suggesting that TSH could either affect purine ribonucleotide formation, RNA synthesis or both. The investigations on the action of TSH on RNA synthesis using isolated sheep-thyroid nuclei indicate that TSH stimulates RNA synthesis from nucleoside triphosphate precursors (Table 14). TSH stimulated the uptake of labelled UMP from UTP into RNA in the presence of the

other three ribonucleoside triphosphates by 69% of the control value. This is similar to the magnitude of increase in adenine uptake observed when the nuclei were incubated with (^{14}C) adenine and TSH. However, when the nuclei were incubated with (^{14}C) adenine in the presence of equimolar amounts of the four ribonucleoside triphosphates, the increment in adenine uptake caused by TSH was 33% of the control value (Table 14). Thus, since addition of triphosphates lowered but did not abolish the stimulatory action of TSH on (^{14}C) adenine uptake into RNA, it appears that TSH may have an effect both on nucleotide formation as well as on RNA synthesis from nucleoside triphosphate precursors. Thus from these observations on the effect of TSH on thyroid RNA metabolism in vitro, it appears that TSH affects RNA synthesis and also perhaps purine nucleotide formation.

Puromycin added along with TSH to isolated nuclei was found to inhibit the effect of added TSH on adenine uptake into RNA from 10 min. incubation onwards (Tables 16 and 17); the uptake of (^{14}C) leucine into nuclear protein was inhibited by puromycin to an extent of about 60% at this time in both

control and TSH-treated nuclei (Table 15). Although puromycin treatment inhibited the action of TSH on nuclear RNA metabolism, suggesting that synthesis of some nuclear protein fraction is essential for this effect, there was no detectable effect of TSH on (^{14}C) leucine uptake into nuclear protein at times where increased adenine uptake into nuclear RNA due to TSH was demonstrable (Fig. 22). This suggests that the protein involved in stimulation of nuclear RNA synthesis by TSH must contribute to only a small proportion of the total nuclear protein. However the possibility must be considered that puromycin may be acting on thyroid nuclear RNA synthesis by means other than inhibition of protein synthesis since, as mentioned on p. 33, puromycin is rather toxic and may have other sideeffects in vitro.

If it is assumed that the puromycin effect is on the synthesis of some nuclear protein, it is of interest to consider the possible nature and function of the protein or proteins involved. Since synthesis of this protein fraction is necessary for the stimulatory action of TSH on thyroid RNA synthesis, it could perhaps be an RNA polymerase or polymerases. TSH could either cause the synthesis of more of an already existing polymerase enzyme or

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enzymes or cause the production of a new polymerase. From studies on bacterial RNA and protein synthesis using the inhibitor of protein synthesis chloramphenicol, it has been suggested that synthesis of some protein is necessary for the regulation of ribosomal RNA synthesis in bacteria (Kurland and Maalke, 1962; Maalke and Kurland, 1963). The nature of this protein is not known at present but it could presumably be either a polymerase enzyme necessary for the synthesis of ribosomal RNA or some protein that is itself subsequently incorporated into bacterial ribosomes. If a similar situation exists in mammalian nuclei, and if the synthesis of the protein which regulates ribosomal RNA synthesis is inhibited by puromycin, an effect of TSH on ribosomal RNA synthesis will no longer be demonstrable once the nuclear supply of this protein is exhausted.

(c) Studies on DNA-Dependent RNA Polymerase Activity

The studies carried out on the effect of TSH on the DNA-dependent RNA polymerase activity of enzyme fractions prepared from sheep-thyroid nuclei have been rather preliminary in nature. Direct addition of TSH at the start of

incubation to assay mixtures containing these polymerase enzyme fractions had no stimulatory effect on the DNA-dependent RNA polymerase activity of these fractions (Tables 19 and 24). In connection with this, Dahmus and Bonner (1965) have shown that direct addition of cortisol to chromatin prepared from the livers of adrenalectomized rats did not alter the template capacity of the chromatin for DNA-dependent synthesis of RNA. However, they also isolated the chromatin after in vivo treatment with cortisol, and demonstrated that the template capacity of the chromatin was altered after hormone treatment in this case.

Treatment of isolated sheep-thyroid nuclei with TSH under conditions where it is known to stimulate the uptake of (^{14}C) adenine into nuclear RNA was found to increase the ability of the "Ramuz" aggregate enzyme preparation to incorporate labelled UMP from UTP but not labelled AMP from ATP into RNA (Tables 26 and 28). Since the "Weiss" enzyme fraction was not responsive to TSH in catalysing an increased uptake of UMP from UTP into RNA (Table 25), this suggests that TSH treatment of the nuclei

affects some factor essential for stimulation of UMP uptake into RNA and that this factor is discarded during preparation of the "Weiss" enzyme fraction at the stage of KCl treatment (Fig. 25). It appears that TSH may affect the DNA-dependent synthesis of RNA catalysed by the "Ramuz" enzyme fraction, but the fact that the enzyme preparation is impure and may possess other enzyme activities cannot be neglected. For example, this enzyme fraction may catalyse the incorporation of nucleotide residues from nucleoside triphosphates into RNA by other means, such as terminal addition of AMP or UMP to already existing RNA molecules or by the formation of homopolymers e.g. poly A or poly U. As mentioned already, in Section III, TSH treatment of the nuclei could result in increased DNA-dependent synthesis of RNA catalysed by the "Ramuz" enzyme fraction, and the fact that TSH is apparently not affecting the incorporation of AMP from ATP into RNA may be due to the presence of a poly A-synthesizing enzyme or enzymes, which are not responsive to TSH treatment, and which account for a considerable proportion of the ATP incorporation into RNA. In connection with this, there is

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evidence that enzymes which catalyse the formation of poly A are present in mammalian nuclei (Chambon, Weill and Mandel, 1963; Edmonds and Abrams, 1963). However, even if this were a genuine effect of TSH on DNA-dependent synthesis of RNA, it is not known whether it is due to synthesis of polymerase enzyme, change in activity of the enzyme or in availability of the DNA template. since the "Ramuz" enzyme fraction from sheep-thyroid nuclei contains both the polymerase enzyme and the DNA which serves as template for the reaction. The studies carried out using puromycin, to try to assess whether synthesis of a polymerase enzyme is involved in TSH stimulation of adenine uptake into nuclear RNA, have been unsuccessful so far, but the data in Table 27 suggest that this is not the case. Purification of the "Ramuz" enzyme fraction or preparation of chromatin in such a way as to keep structural damage minimal is necessary before the effect of TSH on thyroid DNA-dependent RNA polymerase activity can be satisfactorily tested.

Thus TSH affects thyroid RNA metabolism by increasing the synthesis of nuclear RNA, which is followed by an increase in

labelling of cytoplasmic RNA. This apparently, from in vivo studies, results in an increase in general thyroid protein synthesis (Ekholm and Pantić, 1963). This effect of TSH on thyroid RNA metabolism was not demonstrable at times earlier than 1 hr. during incubation of sheep-thyroid slices, but was evident within 20 min. of adding TSH to isolated sheep-thyroid nuclei in vitro. The synthesis of both nuclear protein and RNA appears to be necessary for the effect of TSH on thyroid nuclear RNA metabolism. The protein involved must contribute to only a very small proportion of the total nuclear protein, but the nature of the protein has not been elucidated as yet. It may be an RNA polymerase enzyme or else some protein component of thyroid nuclear ribosomal particles. These results, obtained using isolated sheep-thyroid nuclei, of the effect of TSH on RNA metabolism are probably genuine, since the in vivo studies of Greenspan and Hargadine (1965) have shown that TSH enters the nuclei of thyroid epithelia. However, the possibility of artifacts arising from loss of cellular control by destruction of certain subcellular arrangements during nuclear preparation should not be disregarded.

Comparison of TSH Effects on Various Thyroid Metabolic Processes

The reported actions of TSH on various thyroid metabolic processes have already been described in detail in the General Introduction, and therefore discussion will be limited to those effects which occur very soon after TSH administration and which also appear to be specific actions of TSH on the thyroid gland. It has been shown that TSH stimulates the oxidation of glucose via the hexose monophosphate pathway within 5 min. of addition to thyroid slices in vitro (Field et al., 1960; Dumont, 1961). This appears to be a specific effect of TSH on the thyroid gland (Field et al., 1960; Dumont, 1964), and occurs using physiological levels of TSH (Dumont, 1961). This TSH-mediated increase in glucose oxidation appears to be secondary to an increased conversion of NAD to NADP (Pastan, Herring and Field, 1961). These effects are not inhibited by treatment of thyroid slices with actinomycin D or puromycin which prevent DNA-dependent RNA synthesis or protein synthesis (Field, Johnson, Kendig and Pastan, 1963; Dumont and Burelle, 1964; Field, Epstein and Jarrett, 1965).

Thus for the action of TSH on glucose oxidation, no de novo synthesis of RNA or protein seems to be required. This increased oxidation of glucose is also not apparently due to increased permeability to glucose (Field et al, 1960). Another effect of TSH which can be detected very soon after treatment is proteolysis of thyroglobulin and release of thyroid hormone from the gland. This effect is not inhibited by treatment with puromycin to block protein synthesis (Tong, 1965), and is still demonstrable when organification of iodine is inhibited (Tong, 1964). It has been proposed that the effect of TSH on breakdown of thyroglobulin resulted in oxidation of glucose to provide energy for this process. However, breakdown of thyroglobulin itself has been ruled out as a necessary feature of stimulation of the thyroid by TSH (Taurog, Tong and Chaikoff, 1958b; Pastan, 1961). The thyroid secretory process also involves pinocytosis of colloid (Nadler, Sakar and Leblond, 1962), and the hypothesis that the enhancement of this process is a primary effect of TSH has not been disproved. The time element is also important in attempting to assess a primary target of TSH action. The measured speed at which TSH appears to affect any thyroid

metabolic process is dependent on the sensitivity of the method of detection used. Since the sensitivities of the procedures used for detection of TSH effects on different thyroid metabolic reactions are variable, it is not possible to put the reported actions of the hormone on thyroid metabolism in their correct sequence. A comparison of these reported actions of TSH with studies of the effect of TSH on thyroid nucleic acid metabolism, suggest that, on the basis of the investigations using puromycin and actinomycin D, these effects may not all be attributed to a single action of TSH. It may be that TSH has more than one site of action in the thyroid gland.

General Considerations of Hormonal Control of Protein Biosynthesis

The effects of TSH on the thyroid, which are similar to those of other hormones, such as growth hormone and insulin on their target organs (Kerner, 1965b), can be arbitrarily separated into two categories from studies using inhibitors of RNA and protein synthesis: (a) the effects on enzyme activity which apparently do not require new synthesis of RNA, and (b) the effects that do result in the synthesis of more RNA

or new RNA. Although these categories would seem to be distinct, they are somewhat interwoven. Potter (1965) has drawn attention to the possibility that there may be enzyme formation without RNA synthesis immediately preceding it. In addition, because of the state of the α -protein synthetic mechanism in the unstimulated cell cytoplasm RNA synthesis may not be maximal and a hormone may affect this state resulting in increased RNA synthesis.

Monod (1966) has shown in a study of allosteric mechanisms that, within the living cell, the compounds used most widely as allosteric ligands, that is as chemical signals to control intracellular metabolism, are themselves intermediary products of this metabolism. However, these compounds do not participate in the reactions which they control. Also from studies of bacterial metabolism it has been shown that the same metabolite may act simultaneously at several different levels of cellular control (Monod, 1966). The function of hormones is to serve as chemical signals between different tissues and organs, and in order to influence the metabolism of the cells of their target organs, they must

act directly or indirectly to modify certain enzymic activities. Thus perhaps a hormone could act at the level of control of protein synthesis or as an allosteric effector of an enzyme system, i.e. act simultaneously at several different metabolic points. However, the concept of a polypeptide hormone, such as TSH, acting as an allosteric effector is somewhat difficult to accept at the moment, since all the compounds which are known to have allosteric effects on enzyme molecules are ones of low molecular weight, and there is no evidence so far of a protein-protein interaction producing an effect of this type.

Also little is known of the mechanisms involved in the target specificity of a hormone or the mode of inactivation of a hormone after it has affected the metabolism of its target tissue. Drawing an analogy from control in bacterial systems, perhaps hormones induce their own means of inactivation by some intermediate or end-product of the reaction(s) which they stimulate. For instance the effect of TSH results in the production of thyroid hormones which have to pass through the thyroid epithelia before their

release into the circulation, and in doing so could cause the cessation of TSH action. However there is no evidence to show that this type of mechanism exists, and TSH may in fact be continuously inactivated by some mechanism which is intrinsic in the thyroid cells themselves.

At the present time there are many reports of hormones influencing protein biosynthesis in their target organs by apparently affecting (a) the genes, (b) the enzyme-forming systems and (c) the enzyme proteins themselves, but due to the complex nature of the feed-back mechanisms involved in the regulation of protein synthesis at the intracellular level, in no case has a clear-cut action of a hormone on protein biosynthesis been demonstrated. The reason for this is that little is known of the basic mechanisms operative in controlling protein synthesis in mammalian cells, and current concepts of regulation are mainly drawn from those which have been shown to be operative in bacterial systems. Therefore in order to study the action of a hormone, such as TSH, on the protein biosynthetic mechanism of its target organ, some knowledge of the basic mechanisms involved in the intracellular control

of protein biosynthesis in that organ, would perhaps facilitate the elucidation of the mechanism of action of the hormone on cellular protein synthesis.

Conclusions

From studies using isotopically-labelled RNA precursors, it has been shown that TSH affects thyroid RNA metabolism by increasing the synthesis of nuclear RNA which is followed by an increase in labelling of cytoplasmic RNA. The effect of TSH on nuclear RNA metabolism was not demonstrable at times earlier than 1 hr. during incubation of sheep-thyroid slices but was evident within 20 min. of adding TSH to isolated sheep-thyroid nuclei in vitro. Treatment with actinomycin D inhibits this action of TSH, suggesting that DNA-dependent synthesis of RNA is involved. From investigations using puromycin it also appears that synthesis of nuclear protein is essential for the action of TSH on thyroid RNA synthesis. The protein fraction involved must be a very small proportion of the total nuclear protein, but it has not been characterized as yet. Treatment of isolated nuclei with TSH also resulted in an increase in the

uptake of UMP from UTP into RNA catalysed by a DNA-dependent RNA polymerase fraction prepared from the nuclei. This suggests that the protein fraction mentioned above may be a DNA-dependent RNA polymerase. However, when ATP replaced UTP during polymerase assay after TSH treatment, no stimulation of uptake of AMP from ATP into RNA was observed. In the light of current concepts of control of cellular protein synthesis, the cell nucleus as the site of action of a hormone is very strategic, but whether these reported effects of TSH on the thyroid cell nucleus reflect those occurring in the intact animal remains to be elucidated.

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